ANALYSIS OF CEPHALOSPORIN ANTIBIOTICS

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CERTIFICATE

This is to certify that the thesis entitled " ANALYSIS OF CEPHALOSPORIN ANTIBIOTICS" submitted in fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY (ANALYTICAL CHEMISTRY) is a record of bonafide research carried out by Pritesh Rameshbhai Upadhyay at Lupin Research Center, Mandideep and Lupin Research Park, Pune, under our supervision and the manuscript is suitable for submission for the award of degree of DOCTOR OF PHILOSOPHY IN ANALYTICAL CHEMISTRY.

This is to further certify that Pritesh Rameshbhai Upadhyay has put in minimum 200 days attendance in the Department of Analytical during the course of this study.

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(P.R.UPADHYAY)

CONTENTS

CEPHALOSPORIN

Introduction: -

Cephalosporins C is the parent substance from which the first cephalosporins to find clinical use were derived. Cephalosporin C is produced in very small amounts by a wild strain of a species of Cephalosporium [Commonwealth Mycological Institute Kew (C.M.I.) NO. 49137], similar to Cephalosporium acremonium, which was isolated by Brotzu. Mutants of this strain have been obatined which produce Cephalosporin C in much higher yield. Certain strains of Emericellopsis terricola var. glabra may also produce small quantities of this antibiotic.[1]

In addition to Cephalosporin C, the Cephalosporium sp. C.M.I. 49137 produces Cephalosporin N, which is related chemically to Cephalosporin C, and an entirely different antibiotic, Cephalosporin P.[2, 3,4] Cephalosporin N, now known as penicillin N, is identical with an antibiotic which was formerly named synnematin B[5]. Work which began with the study of the later product has shown that the ability to produce penicillin N is shared by a number of different fungi.

In 1943 certain members of the Fusarium-Cephalosporium group were found by Waksman and Horning to be antagonistic to the growth of bacteria on a solid medium. Eight years later Gottshall et al [6] reported that an antibiotic was produced by a member of the genus Tilachlidium and by Cephalosporium charticola. The Tilachlidium was then found to be a new species of Cephalosporium and it was named C. salmosynnematum.[7] The name Synnematin,

later changed to Synnematin B, was thus given to the antibiotic concerned, whose chemical nature was not then known.[8]

The perfect stage of C. salmosynnematum was observed in 1957 by Grosklags and Swift and this organism was then classified as a new species of the genus Emericellopsis Van Beyma (E. salmosynnematum). Several species of Emericelloipsis were shown to produce penicillin N [9,10] (synnematin), and the later was isolated from the culture fluid of C. Chrysogenum Thirum and Sukapure. [11,12] Only Cephalosporin P was found in culture fluids of E. humicola, and none of these species were reported to produce Cephalosporin C. Penicillin N has also been reported to be formed by a member of the genus streptomyces[13] and by Paecilomyces persicinus.[14] However, the configuration of the α-aminoadipic acid residue in the products from these organisms does not appear to have been determined.It has been suggested by Mangallam et al.[15] that the organism isolated by Brotzu should be classified as a strain of C. Chrysogenum and by Gams[16] that it should be described as a strain of Acremonium chrysogenum.

Development :-

After the demonstration at Oxford of the chemotherapeutic properties of penicillin, a search for antibiotic - producing organisms was made by Giluseppe Brotzu in Sardinia. Brotzu began this work in July of 1945, and has examined the microbial flora of seawater near a sewage outlet at Cagliari, supposing that the process of self-purification of the water might be due in part to bacterial antagonism. From a spot which is now reclaimed land he isolated a fungus which

he concluded was similar to C.acremonium. When grown on agar this organism secreted material that inhibited the growth of a variety of gram-positive and gram-negative bacteria. Selection of colonies from hundreds of serial cultures on agar plates led to the isolation of a strain which produced significant amounts of antibiotic material when grown in glucose-starch broth. From the filtrates of such cultures a crude active concentrate was obtained after precipitation of inactive products with ethanol.

Both culture filtrates and crude active concentrates from the Cephalosporium sp. were tested clinically in Sardinia. The filtrates were injected directly in to staphylococcal and streptococcal lesions, particularly boils and abscesses, with results that were reported to be good. The concentrates were given intravenously and intramuscularly to patients with typhoid fever, paratyphoid A and B infections, and brucellosis. Although the treatment was complicated by pain and by pyrogenic effects, it usually appeared to produce marked improvement, particularly in cases of typhoid fever. Brotzu believed that his results offered hopeful prospects, but he concluded that isolation of the active principle would be beyond his resources and expressed the hope, at the end of his publication, that the work would be taken up elsewhere.

The initial experiments at oxford with the Sardinian Cephalosporium sp. (C.M.I. 49137) were carried out by N.G.Heatley, who found that culture fluids contained an acidic antibiotic which was readily extractable into organic solvents. After July 1949 the production of culture fluids was carried out and at the same time the study of the

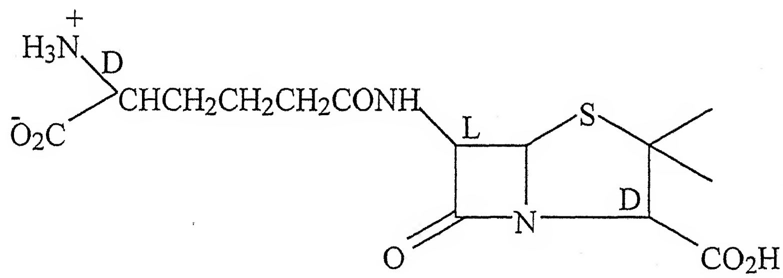
active material was undertaken by H.S.Burton and E.P.Abraham. Attention was first concentrated on the antibiotic extractable into organic solvents, but it became clear that this substance, which was called Cephalosporin P because it showed activity only against certain gram-positive bacteria, was not the antibiotic described by Brotzu.

In August 1949 a second antibiotic was found in Oxford to be present in the culture fluid of the Cephalosporium sp. This substance remained in the aqueous phase after the extraction of cephalosporin P and was discovered independently at Clevedon. It was active against gram-negative as well as gram- positive bacteria and was named Cephalosporin N.[17,2] There appeared to be little doubt that Cephalosporin N was responsible for the antibacterial activity that had first been observed 4 years earlier in Sardinia.

The first clear evidence that Cephalosporin N was a new type of Penicillin was obtained early in 1952, when E.P.Abraham and G.G.F.Newton showed that a partially purified sample of the antibiotic yielded the characteristic amino acid Penicillamineine (β,β-dimethylcysteine) on acid hydrolysis.

At this stage it was decided to increase the effort to isolate and characterize Cephalosporin N. It was found that the culture medium became deficient in methionine during the fermentation. Addition to the medium of methionine, in particular the D isomer, was then shown to increase the yield of Cephalosporin N,[18] and following the resulting improvement in supply, this antibiotic was finally isolated by Abraham et al.[19] in a form that was nearly pure.

Cephalosporin N yielded D-α-aminoadipic acid as well as penicillamine on acid hydrolysis.[3] Further studies left no doubt that it had the structure(I) with a residue of D-α-aminoadipic acid linked through its δ-carboxyl group to the nucleus of the penicillin molecule.[20] It was subsequently renamed penicillin N.



(I)

Chemical Studies

The prospects of obtaining Cephalosporin C in relatively large amounts were much improved when a search for higher - yielding mutant strains of the Cephalosporium sp., undertaken in 1957 by B.K.Kelly and his colleagues at the Antibiotics Research Station, Clevedon, began to be rewarding. Mutant 8650, which produced much more Cephalosporin C than wild strain, was used in subsequent fermentations. This eventually led to a supply of antibiotic from Clevedon which was sufficient for its chemical structure to be determined.

Progress in studies on the chemical degradation of Cephalosporin C enabled a structure to be proposed for this substance at Oxford, in April 1959, which was later confirmed by further chemical work[21]and by an X-ray crystallographic analysis.[22] Attention was then turned by Newton and Abraham with Bronwen Loder, to the possibility of obtaining the nucleus of the Cephalosporin C molecule, stimulated by a report published by Batchelor et al.,[23] of the characterization and isolation in quantity of the Penicillin nucleus, 6 - aminopenicillanic acid. Since Cephalosporin C had the same D-α-aminoadipyl side chain as the penicillinase-sensitive penicillin N, it appeared that its resistance to staphylococcal penicillinase depended on its ring system, and a comparison of the antibacterial activities of penicillin N and benzylpenicillin suggested that appropriate changes in the side chain of cephalosporin C would lead to compounds with much higher activities, at least against gram-positive bacteria. The relative stability of cephalosporin C ring system enabled the later to be obtained in very low yield, by mild acid hydrolysis of cephalosporin C. A small amount of this substance, 7-aminocephalosporanic acid, had been isolated in a relatively pure state and its N-phenylacetyl derivative had been shown to be much more active than cephalosporin C against a penicillinase-producing strain of S.aureus.[24] However, the problem of producing 7- aminocephalosporanic acid in quantity remained to be solved.

Several pharmaceutical companies expressed interest in the cephalosporins at a relatively early stage. The Distillers Company (Biochemicals) Ltd. made contact with the Oxford workers through

Sir Howard Florey in 1954 and considered the possibility of providing a supply of penicillin N. In 1955 Imperial Chemical (Pharmaceuticals) Ltd. initiated a personal connection with the object of becoming acquainted with the current research on penicillin N and cephalosporin C. In the same year an informal suggestion came to Oxford from Eli Lilly and Company that a liaison might be arranged for the purpose of producing Cephalosporin C. Glaxo also showed serious interest.

In 1956 N.R.D.C. began to organize meetings between members of Glaxo's staff and research workers at Oxford and Clevedon. The difficulty of producing cephalosporin C in any quantity from low - yielding Cephalosporium sp. Slowed the pace of further development. However, after the higher - yielding mutant 8650 had become available in 1957, 100gm of cephalosporin C was ultimately produced in the Glaxo Laboratories and some of this material was used in the last experiments made to confirm the chemical structure which had already been proposed.

In 1958 A.M.Van Arendonk, director of Eli Lilly patent division, approached the National Research Development Corporation and discussed with B.J.A.Bard and J.C.Cain a proposal for a program aimed at the production of substances related to cephalosporin C, including the nucleus of the molecule, by fermentation. This idea stemmed from work by E.H.Flynn and his colleagues, which was then under way, on the isolation of the penicillin nucleus, 6 - aminopenicillanic acid. Eli Lilly signed an agreement with the Corporation in January 1959. Although the project concerned was

not successful, they entered into a general option agreement early in 1960 under which they received mutant 8650 of the cephalosporium sp. and access to technical information. From then on they were to make increasingly important contributions to the cephalosporin field.

Other pharmaceutical companies were now showing interest in the cephalosporins. A general option for a license had been obtained from N.R.D.C. by E.R.Squibb and Company in 1959. In addition to Eli Lilly, three U.S.companies, Merck and Company, Chas. Pfizer and company, and Smith Kline and French, entered into option agreements in the following year, as did CIBA in Switzerland and Farmitalia in Italy. In 1961 a similar agreement was made with the Fujisawa Pharmaceutical Company in Japan. Until this time it had appeared possible that cephalosporin C itself might find some clinical use for treatment of penicillin-resistant staphylococcal infections, even though its very low specific activity would presumably have required it to be given by intravenous infusion. But this appeared unlikely after the production of 2,6 - dimethoxyphenylpenicillin(methicillin) from 6 - aminopenicillanic acid and the demonstration of its Chemotherapeutic properties.[25,26] Thus, a great deal depend on the discovery of a method for the production of 7- aminocephalosporanic acid on a large scale.

Extensive searches in several laboratories for an enzyme which would remove the D - α - aminoadipyl side chain from cephalosporin C had no significant success. But before the end of 1960 an ingenious chemical procedure had been discovered in the Lilly Research Laboratories which enabled the side chain to be removed

and 7-aminocephalosporanic acid to be obtained in very much higher yield than was possible by simple hydrolysis[27]. Detailed of an improved version of the original process were reported to the N.R.D.C. By this time work at Eli Lilly and at Glaxo had opened the way to the production of cephalosporin C in large amount by fermentation. Thus, 7-amonocephalosporanic acid became available in quantity and an intensive study of the properties of derivatives of the compound soon led to the introduction of two cephalosporins, namely cephalothin and cephalorodine, in to medicine.

In comparison with that of many other antibiotics the first stages of history of cephalosporins were unusual and prolonged. Eight years elapsed between the isolation of the Cephalosporium sp. in Sardinia and the discovery of cephalosporin C in Oxford, largely because the activity of this substance in a conventional assay. A further 7 years passed before the isolation of high-yielding mutant strains of the organism and the discovery of a novel method for obtaining the nucleus of the molecule allowed the potentialities of the new ring system of the latter to be adequately explored. During this latter period the difficulties to be overcome appeared at a times to be so formidable that it would not have been surprising if the project had been abandoned. Its final success must be attributed to a combination of scientific ability, technical expertise and willingness to take calculated risk in the pharmaceutical companies that were mainly involved.

PENICILLINS AND CEPHALOSPORINS

Comparison

While a number of Penicillanic acids are produced in fermentations of Penicillium fungi, β-lactam antibiotics from other microorganisms are obtained as derivatives of α-aminoadipic acid. Although these compounds have not been of clinical use per se, comparison of cephalosporin C and penicillin N has historical significance. Chemical modification of these naturally occurring substances has provided compounds which indicate the types of structural variation capable of producing more potent antibiotics. One example of this, already noted, is the conversion of cephalosporin C to cephalosporin C_A by reaction with pyridine. More recently, several streptomycetes have been shown to produce β-lactam antibiotics closely related to those produced by fungi. The first reported instance of β-lactam production by a streptomycete was the isolation of penicillin N in the Merck Laboratories[28]. Other β-lactams originating from streptomyces species are those reported by Nagarajan et al.[29] They include a cephalosporin having a C-3 carbamoyloxymethyl moiety, another which has a methoxyl in place of hydrogen at C-7, and a third having both of the foregoing substituents in the same molecule.

The antibiotic activity of β-lactam antibiotics which occur in nature as compared to cephalothin. Several derivatives prepared by reaction carried out at the 3-methyl position are included to indicate trends observed in early work on these compounds by Abraham[30] . Both penicillin N and cephalosporin C have less than 1% of the activity of

benzylpenicillin against a penicillin-sensitive staphylococcus[31], but they show superior activity against gram-negative organisms, with penicillin N being more potent in several cases and cephalosporin C in others. The penicillin is more active against a penicillin-sensitive S. aureus (3055) where 1.0 mg/ml solutions of each produced zone diameters of 18 and 14 mm, respectively. The replacement of the acetoxyl function (cephalosporin C) on the 3-methyl group by a primary carbamate produces little change in in-vitro antibacterial activity. Within the limits of the test these compounds appear equivalent. In the α-aminoadipic acid- containing antibiotics the presence of a methoxyl function at C-7 results in diminished activity against the gram-positive microorganisms. In contrast, a significant increase in inhibitory properties toward gram-negative bacteria is observed. The qualitative gram-negative bacterial spectrum of these methoxy-substituted antibiotics is almost the same as for cephalosporin C.

The reaction of 7-ACA derivatives with nucleophiles has been studied extensively, and many examples of these products have been cited. The reaction of cephalosporin C with pyridine was reported by Hale et al.[31] In contrast to most variations of structure, the resulting pyridinium betaine shows enhancement of both gram-negative and gram-positive activity.

A more common pattern observed when looking at new β-lactam antibiotic derivatives is a shift in antibacterial spectrum. For example, the 7-methoxyl cephalosporin C derivative shows a decrease in activity toward gram-positive organisms along with the increase in

activity against the gram-negative bacteria. This shift in antimicrobial spectrum appears to be consistent with correlation mentioned earlier concerning the differences in lipophilic character of the cell walls of various bacteria.

These changes in biological spectrum of the α-aminoadipoyl derivatives are strongly suggestive of effects observed also with other 7-amido functions and have therefore been of great predictive value in structure-activity studies.

One may generalize that in non-β-lactamase-producing gram-positive bacteria the penicillin will be more inhibitory. Chauvette and co-workers commented on this fact very early while working with cephalosporins which, when directly compared in Oxford units of activity, were only about a fifth as active as the corresponding penicillin. They found that structural requirements for high activity were similar for side-chain amides of cephem and penam antibiotics. The difference in relative levels of inhibition by penam as compared with the cephem[32] derivatives. Available evidence, cited earlier, attributes this intrinsic difference to a greater chemical reactivity of the β-lactam in the penicillin ring system.

The situation is more complex when comparing the activity of penicillanic and cephalosporanic acid toward gram-negative bacteria. The occurrence of β-lactamases is more common and their specificity has been shown to be more divers in gram-negative organisms[33]. A penicillin β-lactamase since inhibition by the

penicillins listed is uniformly poorer than for the cephalosporins, while less differences is evident when E.coli N10 is considered. Against many gram-negative organisms the MIC values are equivalent. Rarely, however, does the penicillin exhibit a superior level of activity. Serratia marcescens (X99) is one of these exceptions and generally responds to penicillins at lower MIC values than to cephalosporins, i.e., a penicillin (ampicillin) MIC of 8.8 μg/ml and a cephaloglycin MIC of 68 μg/ml are found for X99.

Naito and co-workers have compared several derivatives of sydnone-3-acetamido-substituted penicillins and cephalosporins and find a broad spectrum of biological activity in both the penam and cephem compounds. Again, the penicillins are somewhat more active against gram-positive bacteria and less active against the gram-negative organisms used. Stedman et al. Investigated a series of pyridylacetamidocephalosporanic and penicillanic acid which also conformed to these generalizations. However, Raap and Micetich found consistently higher activity for a series of substituted isothiazolylmethylpenicillins over the corresponding cephalosporins using strins of E.coli and several Salmonella. MIC values were determined with a twofold serial dilution technique.

The effects of penicillin β-lactamase-resistant penicillins are evident from the dramatic drop in MIC values with the resistant staphylococcus organisms cited when they are exposed to methicillin or oxacillin. Price states that these compounds have only 2 and 10 % respectively of the activity of the penicillin G against sensitive staphylococci. S. aureus X400 is resistant to all penicillins

including methicillin,and this strin whose resistance is not well understood is also resistant to most cephalosporin antibiotics.

The beta-lactam family of antibiotics includes many of the most heavily used antibacterials in clinical medicine. They are important, both historically and currently, because of their effectiveness and generally low toxicity. The beta-lactam structure is being exploited by many drug development groups in the search for new drugs with improved efficacy against resistant strains of bacteria.

The majority of the clinically useful beta-lactams belong to either the penicillin (penam) or cephalosporin (cephem) group. Moxalactam is an antibacterial that belongs to the oxacephem group which is closely related to the cephalosporins. For convenience, it is common to include it as a member of the cephalosporins because its pharmacology is so closely related to the so-called third generation cephalosporins. The beta-lactams also include the carbapenems (e.g., imipenem), the monobactams, e.g., aztreonam, and the beta-lactamase inhibitors, (e.g., clavulanic acid).

Cephalosporins are semisynthetic antibiotic derivatives of cephalosporin C, a substance products by the fungus Cephalosporium acremonium. All commercially available cephalosporins contain the 7 - aminocephalosporinic acid (7-ACA) nucleus which is composed of a β- lactam ring fused with a 6-membered dihydrothaizine ring. Addition of various groups at R1 (position 7) and R2 (position 3) of cephalosporin nucleus results in derivatives with differences in spectra of activity, stability against

hydrolysis byβ-lactamases, protein binding, GI absorption, or susceptibility to desacetylation.

Currently available cephalosporins are generally divided into 4 groups based on spectra of activity:

- first generation cephalosporin
- second generation cephalosporin
- third generation cephalosporin and
- fourth generation cephalosporin

First generation cephalosporins

First generation cephalosporins usually are active in vitro against gram-positive cocci including penicillinase producing and nonpenicillinase producing Staphylococcus spp. First generation cephalosporins have limited activity against gram-negative bacteria that they are inactive against enterococci (eg. Enterococcus faccalis), methicillin- resistant staphylococci, Bacteroides fragilis, Citrobactor, Listeria monocytogenes, Proteus other than P. mirabilis, Providencia, Pseudomonas and Serratia. Example Cefadroxial, Cefazolin, Cephalexin, Cephapirin, Cephradine and Cephalothin.

Second generation cephalosporins

Second generation cephalosporins usually are active in vitro against bacteria susceptible to first generation drugs are active in vitro against most strains of Haemophilus influenzae concluding

ampicillin-resistant strains) and more active in vitro against gram-negative bacteria than first generation cephalosporins.Example Cefaclor, Cefamandole, Cefmetazole,Ceforanide, Cefotetan, Cefoxitin, Cefprozil, Cefuroxime axetil and Loracarbef.

Third generation cephalosporins

Third generation cephalosporins usually are less active in vitro against susceptible staphylococci than first generation cephalosporins; however, the third generation drugs have an expanded spectrum of activity against gram-negative bacteria compared with the first and second generation drugs and also are active in vitro against Citrobacter, Enterobacter, E.coli, Klebsiella, Proteus , Morganella , Providencia and Serratia that may be resistant to first and second generation cephalosporins . Some parenteral third generation drugs have activity in vitro against B.fragilis and Pseudomonas. Third generation cephalosporins are inactive against most slaphylococci , enterococci (E. faccalis) and L. monocytogenes. Example Cefdinir, Cefixime, Cefoperazone, Cefotaxime, Cefpodoxime, Ceftizoxime, Ceftriaxone, Ceftazidime and Ceftibuten.

Fourth generation cephalosporins

Fourth generation cephalosporins, like third generation cephalosporins, have an expanded spectrum of activity against gram-negative bacteria compared with the first and second generation drugs. However, fourth generation cepharosporins are

active in vitro against some gram-negative including Pseudomonas aeruginosa and certain Enterobacteriaceae that generally are resistant to third generation cephalosporins. In addition, fourth generation cephalosporins may be more active against gram-positive bacteria than some third generation drugs. The extended spectrum of activity of fourth generation cephalosporins is related to the fact that the drug penetrates the outer cephalosporins and the fact that the drug is more resistant inactivation by chromosomally and plasmid-mediated β-lactamase than most other cephalosporins. Example Cefepime, Cefpirome.

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CHROMATOGRAPHY

Theory

Few methods of chemical analysis are truly specific to a particular analyte. It is often found that the analyte of interest must be separated from the myriad of individual compounds that may be present in a sample. As well as providing the analytical scientist with methods of separation, chromatographic techniques can also provide methods of analysis.

Chromatography involves a sample (or sample extract) being dissolved in a *mobile phase* (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible *stationary phase*. The phases are chosen such that components of the sample have differing solubility in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

Techniques such as H.P.L.C. (High Performance Liquid Chromatography) and G.C. (Gas Chromatography) use *columns* – narrow tubes packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is

called *elution*. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase.

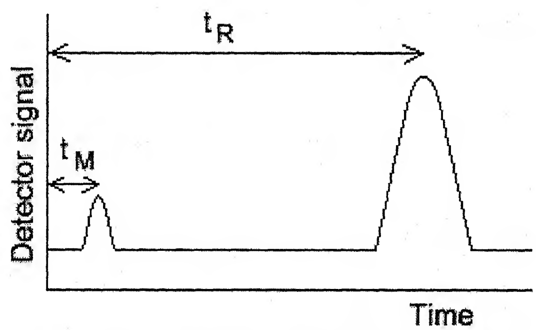
Distribution of analytes between phases

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases;

$$A_{mobile} / A_{stationary}$$

The equilibrium constant, *K*, is termed the *partition coefficient*; defined as the molar concentration of analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase.

The time between sample injection and an analyte peak reaching a detector at the end of the column is termed the *retention time* (t_R).



Each analyte in a sample will have a different retention time. The time taken for the mobile phase to pass through the column is called t_M.

A term called the *retention factor, k'*, is often used to describe the migration rate of an analyte on a column. You may also find it called the *capacity factor*. The retention factor for analyte *A* is defined as;

$$k'_A = t_R - t_M / t_M$$

t_R and t_M are easily obtained from a chromatogram. When an analytes retention factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideally, the retention factor for an analyte is between one and five.

We define a quantity called the *selectivity factor*, α, which describes the separation of two species (A and B) on the column;

$$\alpha = k'_B / k'_A$$

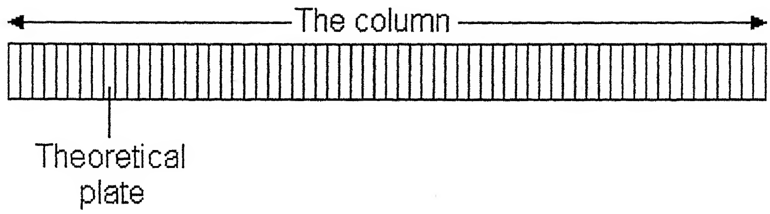
When calculating the selectivity factor, species A elutes faster than species B. The selectivity factor is always greater than one.

Band broadening and column efficiency

To obtain optimal separations, sharp, symmetrical chromatographic peaks must be obtained. This means that band broadening must be limited. It is also beneficial to measure the efficiency of the column.

The Theoretical Plate Model of Chromatography

The plate model supposes that the chromatographic column is contains a large number of separate layers, called *theoretical plates*.



Separate equilibration of the sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.

It is important to remember that the plates do not really exist; they are a figment of the imagination that helps us understand the processes at work in the column. They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, *N* (the more plates the better), or by stating the plate height; the *Height Equivalent to a Theoretical Plate* (the smaller the better).

If the length of the column is *L*, then the HETP is

$$\text{HETP} = L / N$$

The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution;

$$N = \frac{5.55\, t_R^{\,2}}{w_{1/2}^{\,2}}$$

where $w_{1/2}$ is the peak width at half-height.

As can be seen from this equation, columns behave as if they have different numbers of plates for different solutes in a mixture.

The Rate Theory of Chromatography

A more realistic description of the processes at work inside a column takes account of the time taken for the solute to equilibrate between the stationary and mobile phase (unlike the plate model, which assumes that equilibration is infinitely fast). The resulting band shape of a chromatographic peak is therefore affected by the rate of elution. It is also affected by the different paths available to solute molecules as they travel between particles of stationary phase. If we consider the various mechanisms which contribute to band broadening, we arrive at the Van Deemter equation for plate height;

$$\text{HETP} = A + B / u + C u$$

where u is the average velocity of the mobile phase. A, B, and C are factors which contribute to band broadening.

A **- Eddy diffusion**

The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths.

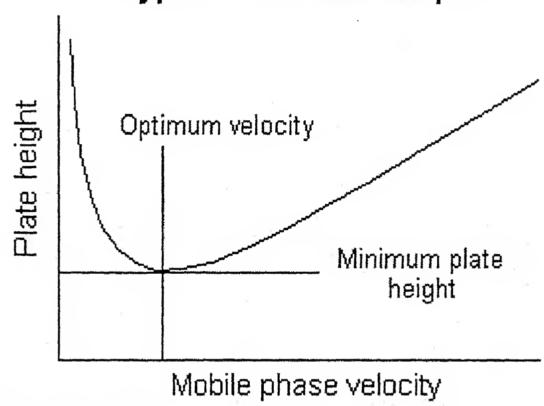
B - Longitudinal diffusion

The concentration of analyte is less at the edges of the band than at the center. Analyte diffuses out from the center to the edges. This causes band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion.

C - Resistance to mass transfer

The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

A typical Van Deemter plot



Van Deemter plots

A plot of plate height vs. average linear velocity of mobile phase.

Such plots are of considerable use in determining the optimum mobile phase flow rate.

Resolution

Although the selectivity factor, α, describes the separation of band centres, it does not take into account peak widths. Another measure

$$R = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

of how well species have been separated is provided by measurement of the *resolution*. The resolution of two species, A and B, is defined as

Baseline resolution is achieved when R = 1.5

It is useful to relate the resolution to the number of plates in the

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

column, the selectivity factor and the retention factors of the two solutes;

To obtain high resolution, the three terms must be maximized. An increase in *N*, the number of theoretical plates, by lengthening the column leads to an increase in retention time and increased band broadening - which may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the size of the stationary phase particles.

It is often found that by controlling the capacity factor, k', separations can be greatly improved. This can be achieved by changing the temperature (in Gas Chromatography) or the composition of the mobile phase (in Liquid Chromatography).

The selectivity factor, α, can also be manipulated to improve separations. When α is close to unity, optimising k' and increasing N is not sufficient to give good separation in a reasonable time. In these cases, k' is optimised first, and then α is increased by one of the following procedures:

- Changing mobile phase composition.
- Changing column temperature.
- Changing composition of stationary phase.
- Using special chemical effects (such as incorporating a species which complexes with one of the solutes into the stationary phase).

Preparative HPLC separation

The main goal of preparative HPLC is the isolation of purified material (product) for further use. When only small amounts of pure product are required, an analytical HPLC method (e.g.,0.46-cm column ID) can be used to recover nanogram-to-sub-milligram quantities of product. The sample band is observed as it passes through the detector, and the band is collected at the detector outlet. Removal of the mobile phase by evaporation or lyophilization then furnished purified product. Large amounts of purified material can be

obtained by injecting larger samples and/or by repetitive injections with pooling of product fractions. A maximum production of purified product per injection is usually a major goal in preparative HPLC. The goal and characteristics of preparative vs. analytical HPLC are as follows.

Analytical HPLC	**Preparatve HPLC**
Information about sample composition.	Recovery of purified product.
Most or all sample components often of interest.	Usually only one or few sample components of interest.
Sample weight just large enough for adequate detection.	Largest possible sample weight for maximum yield of pure product.
Reversed-phase HPLC most convenient.	Normal-phase HPLC most convenient.
Column internal diameter 1-5 mm	Column internal diameter 1-10cm(or large).
Column particles 5.0 μm or smaller.	Column particles 7.0μm or larger
HPLC pumps provide up to 10.0 ml/min	HPLC pumps provide >> 10.0 ml/min.
Sample injection usually not a problem	Sample injection more difficult, requires more attention.
Detection conditions selected for maximum sensitivity.	Detection conditions often selected for reduced sensitivity.
Solubility of sample in mobile phase usually not important.	Sample solubility usually very important.
Mobile-phase volatility unimportant.	Mobile-phase should be volatile; no involatile additives.

Usually, an analytical HPLC procedure will be developed first and can be used as a starting point for a preparative method. Several considerations peculiar to prep LC separation should be kept in

mind, however, first, it will be necessary to remove the mobile phase from collected product fraction(s). For this reason, normal-phase chromatography with organic solvents as the mobile phase is often preferred. The removal of volatile organic solvents from the product will be easier than for reversed-phase HPLC with aqueous mobile phases. Similarly, non-volatile mobile-phase components should be avoided, if possible, making ion-pair chromatography less desirable. If a buffer must be added to the mobile-phase, volatile buffers are preferred(e.g. acetic or formic acid, ammonium carbonate, formate or acetate). Otherwise, a second separation for removal of buffer("desalting") will be required.

A second aspect of prep LC separation is for the separation of crude product from its impurities. When analysis is the goal, baseline resolution of all the bands in the sample is usually desirable. As sample size is increased, the product band will be broaden and quickly overlap the adjacent impurity bands.

A third consideration for prep LC is the solubility of the sample in the mobile phase. Whereas sample solubility is seldom important in developing an analytical method, solubility is often a major factor in prep LC, because it is desirable to inject a large weight of sample dissolved in a relatively small volume of mobile phase. For organic-soluble compounds, normal-phase chromatography usually will be preferred.

A final consideration in prep LC is the relative importance of N and α. As sample size is increased to the point of column overload and

sample band-widths begin to increase, the column plate number becomes more function of a sample size than of column conditions (column length, flow rate, particle size). As a result, it is often advantageous in prep LC to use larger particles and higher flow rates than is the case for analytical separations, because a high column plate number is no longer required. Large values of α are extremely advantageous in prep LC because they allow much larger sample weights.

Method development for a preparative HPLC method should be carried out in the same general way as for an analytical procedure, by adjusting conditions for optimum k,α, and (less important, but significant) N for the product band. As indicated above, normal-phase HPLC will often be a good starting point. If normal-phase conditions are used, unmodified silica is the preferred column packing. Larger (more expensive) columns are often required for preparative separation, and silica is less expensive than polar-bonded-phase packing such as cyano or diol. Using silica, there is also no loss of bonded phase to contaminate collected product fractions (however, silica is somewhat soluble in aqueous mobile phases when the mobile phase pH>6).

METHOD VALIDATION

Introduction

Getting an acceptable separation and detection of compounds is only the first step in a completed method that may be performed for long periods in other laboratories. If the method is used with a product or process, it may be submitted for both internal and official regulatory approval. This could involve agencies such as Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), or their counter-parts around the world.

The transfer of a method is best accomplished by a systematic method validation process. Many workers view validation only as a test of the acceptability of the method using the conditions (e.g.,flow rate, sample size, column type) prescribed. However, the real goal of the validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method. It is important to have a well-conceived validation plań for testing the method and acceptance criteria before starting the validation process. Included in this plan should be detailed procedure describing the entire method (including calibration standard and sample preparation, separation, data handling, and calculations) that can conveniently be executed by others. Many official groups have established guidelines or standard procedures for method validation, and some other recommendations exist in published references[1-7]. However, these guidelines are generally not specific or apply only to certain applications. In this chapter define each of the major items that should be in a good method validation.

Preferred approaches for each phase of a validation process are also given.

General Approach to Method Validation

Just as method development will vary with sample and separation goals, so will method validation. An assay for a major component requires a different approach and acceptance criteria than a method for a trace impurity. The frequency with which a method will be used (many times a day, once a day only for a short study, once a month, etc.) also influences the type of validation studies that are needed.

An iterative approach to overall method validation often is appropriate. The use of a method early in its development may require only limited validation. For example, for initial R&D studies on a new drug candidate, the analyses may performed in a single laboratory, perhaps by one operator on a single instrument. Preliminary toxicology studies on a new pesticide can also be performed under controlled conditions, which minimizes the need for complete validation studies. An HPLC method for an active drug substance used in initial formulation studies may not require a study of detection limit or ruggedness. Therefore, it is best to prioritize the components of validation studies. In a good validation plan the important studies will be done early and anticipate future needs. Typically, specificity, linearity, accuracy, and precision studies are needed first; complete studies of stability and ruggedness often can come later.

A final method may be performed at different sites. Differences in HPLC instrumentation, laboratory equipment, and reagent sources, and variation in the skills and background of personnel may require specific features in the HPLC method. In addition, the development of different formulations of the same drug with varying strengths or physical forms may require flexibility in method procedures. A method developed for the assay of the main component in a tablet may have to be adapted to function in a lotion, cream, or aerosol. The analysis of residual drug in manufacturing equipment (often needed for cleaning - validation studies) also require method modifications. While these types of applications involve method development study. Requirements for validation at a later stages of product development or commercialization may be more stringent, requiring additional studies.

A preferred approach to method validation is to define and carry out the critical studies needed for each step in a manner that allows use of the new and existing information in a subsequent method improvements or validations. In addition, the routine use of a method outside the originating laboratory can provide valuable information on ruggedness(use of different columns, reagents, instruments, etc.). This information from different laboratories should be accumulated during routine use. These later results may indicate that the method should be modified to improve certain characteristics This iterative process continues until a formal, complete validation is performed and documented (usually prior to submission of a drug application, transfer of the final method to a new site, etc.).

The individual components of a method validation study are as follows.

- ACCURACY
- PRECISION
- LINEARITY
- RANGE
- LIMIT OF DETECTION
- LIMIT OF QUANTITATION
- SPECIFICITY
- RUGGEDNESS
- ROBUSTNESS
- STABILITY OF SAMPLES
- REAGENTS
- INSTRUMENTS
- SYSTEM SUITABILITY CRITERIA

For the each component of the study, an important consideration is the need to determine (before the validation starts) What constitutes an acceptable result for that study. These *acceptance criteria* will vary depending on the type of method and its intended use. For example, good precision is more important for an assay of the major component than for a single trace-level impurity.

Accuracy : The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose “true value” is known) is analyzed and the measured value should ideally be identical to the true value.

Typically, accuracy is represented and determined by recovery studies. Accuracy determination for an HPLC method should be carried out with a minimum of nine measurements using at least three concentrations. This approach minimizes any variability and /or bias in sample preparation technique and analysis for one sample at only one concentration. An example would be three replicate measurements each of three different concentration preparations

Precision : Precision can be defined as "the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample"[2].A more comprehensive definition proposed by the International Conference on Harmonization (ICH) [6] de-vides precision into three types namely

- Repeatability
- Intermediate precision, and
- Reproducibility

Repeatability : Is the precision of a method under the same operating condition over a short period of time. This is measured by the sequential, repetitive injection of the same homogeneous sample, followed by the averaging of the peak area value and determination of the relative standard deviation(RSD) of all injections.

Intermediate precision : Is the agreement of complete measurements (including standards) when the same method is

applied many times within the same laboratory. This can include full analysis on different days, instruments, or analysts, but would involve multiple preparation of samples and standards.

Reproducibility : This examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments. Precision is often expressed by the standard deviation (SD) or relative standard deviation (RSD) of a data set. If a set of n measurements is performed on a sample, the average value obtained from those n measurements is defined as

$$X = \frac{\sum_{I=1}^{n} xi}{N}$$

Where xi are the individual measurements on the sample. The standard deviation of these data is then

$$SD = \sqrt{\frac{\sum_{I=1}^{N} (xi - x)^2}{N - 1}}$$

And the relative standard deviation (RSD) or coefficient of variation (CV) is

$$RSD\,(\%) = \frac{100\ SD}{X}$$

Liniearity : The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data are then processed using a linear least-squares regression. The resulting plot slope, intercept, and correlation coefficient provide the desired information on linearity. A linearity correlation coefficient above 0.999 is acceptable for most method, especially for major components in assay method.

Range : The range of a method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity. While a desired concentration range is often known before starting the validation of a method, the actual working range results from data generated during validation studies. For a major component assay, concentrations of standards should be measured at or near the expected target measurement level. The concentration range should encompass values expected in samples to be measured. A good strategy is to perform studies at 50,100,and 150% of target level.

Limit Of Detection : The limit of detection can be defined as the smallest level of an analyte that gives measurable response under stated experimental condition. The LOD is often based on a certain signal-to-noise (S/N) ratio, typically 2 or 3.

Limit Of Quantitation : It can be defined as the smallest concentration of an analyte which gives a response that can be accurately and precisely quantified under stated experimental condition.

Specificity : This can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample materials. Method specificity can be achieved by checking the resolution of all potential interfering compound from the peak of interest.

Robustness : The concept of robustness of an analytical procedure has been defined by the ICH [6] as "a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters." It consists of following parameters.

- Influence of variations in different columns.
- Influence of variations at different column oven temperature.
- Influence of variations at different flow rate.
- Influence of variations at different buffer strength.
- Influence of variations at different pH in mobile phase.
- Influence of variations at different mobile phase composition.

Degradation Study : Degradation of an analyte checked under following stressed conditions.

- Acidic condition
- Alkaline condition
- Oxidative condition
- Thermal condition
- Photochemical condition

Correlate the spectrum of an analyte peak initially and after the experiment, the spectrum correlation value more than 99% indicates that no other peak merge with an analyte peak.

Stability : During the earlier validation studies, the method developer gained some information on the stability of reagents, mobile phase, standards, and sample solution. For routine testing in which many samples are prepared and analyzed each day, it is often essential that solutions be stable enough to allow for delays such as instrument breakdowns or overnight analyses using autosamplers. At this point, the limits of stability should be tested. Samples should be tested over at least a 48 hrs. period, and quantitation of components should be determined by comparison to freshly prepared standards. If the solutions are not stable over 48hrs.,storage conditions or additives should be identified that can improve stability.

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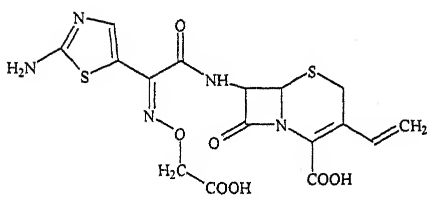
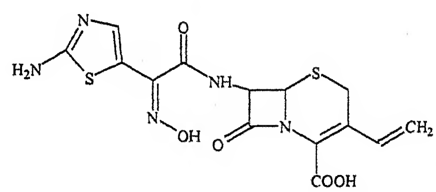
RESEARCH WORK

The purpose of this research work is to study and prepare a process validation protocol requires the identification of critical operating parameters as regards to analytical methods. The critical operating parameter is one, that can impact a product quality attribute, either a specifications parameter or performance characteristic of instruments. The regulatory authorities such as FDA, MCA etc require validation as to the suitability of the allowable ranges for these critical parameters. To carry out present research work, Cefixime and Cefdinir have been selected for the detailed studies of their chromatographic validation and impurity profiling.



Cefixime



Cefdinir

Cefixime and Cefdinir are the first member of what is generally termed the third generation orally active cephalosporins. These third generation cephalosporins are distinct from older β-lactam antibiotics in their intensive antibacterial activity against a wide range of gram-negative bacteria. The exceptional antibacterial activity of third generation cephalosporins has been shown to be based on both

their enhanced affinity for the target enzymes and their high stability to β- lactamase.

Several analytical methods available to analyze these antibiotics such as Chemical, UV and Calorimeter etc. but chromatographic remained the best among all. The advantages of Chromatography are as follows.

- It resolves principal peak from impurities.
- It is accurate
- It is precise
- It is more selective and
- It is Robust

Therefore, High Performance Liquid Chromatography (HPLC) is selected as the method of choice for the analysis of Cefixime and Cefdinir and also extended to study their impurity profile.

In HPLC, getting an acceptable separation and detection of the compound is only the first step in completed method that may be performed for long periods in other laboratories. If the method is used with a product or process, it may be submitted for both internal and official regulatory approval. This could involve agencies such as the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), or their counter-parts around the world.

Many workers view HPLC method validation only as a test of the acceptability of the method using the conditions (e.g. flow rate, sample size, column type) prescribed. However, the real goal of the validation process is to challenge the method and determine limits of

allowed variability for the conditions needed to run the method. It is important to have a well-conceived validation plan for testing the method and acceptance criteria before starting the validation process. Many official groups have established guidelines or standard procedures for method validation, and some other recommendations exist in published references. However, these guidelines are generally not specific or apply only to certain applications.

An assay for a major component requires a different approach and acceptance criteria than a method for a trace impurity. An HPLC method for an active drug substance used in initial studies may not require a study of detection limit or ruggedness. Therefore, it is best to prioritize the components of validation studies. In a good validation plan the important studies will be done early and anticipate future needs. Typically, specificity, linearity, accuracy and precision studies are needed first; complete studies of stability and ruggedness often can come later. Differences in HPLC instrumentation, laboratory equipment, and reagent sources, and variations in the skills and background of personnel may require specific features in the HPLC method.

A preferred approach to method validation is to define and carry out the critical studies needed for each step in a manner that allows use of the new and existing information in subsequent method improvements or validations. In addition, the routine use of a method outside the originating laboratory can provide valuable information on ruggedness. These later results may indicate that the method

should be modified to improve certain characteristics. Method validation study contains following parameters.

- Accuracy
- Precision
- Linearity
- Range
- Limit of detection
- Limit of Quantitation
- Specificity
- Robustness
- Stability of sample and
- Forced degradation

Out of Cefixime and Cefdinir, only former is listed in US and European Pharmacopoeia including its' method of analysis, which may not be necessary to utilize for stability study indicating purpose. Stability indicating method means "The method should be able to resolve main peak from all impurities not only at the time of initial analysis but also at the end of shelf life of the drug". Hence, the method developed initially should have the capability to comply stability indicating parameter is most important part of method.

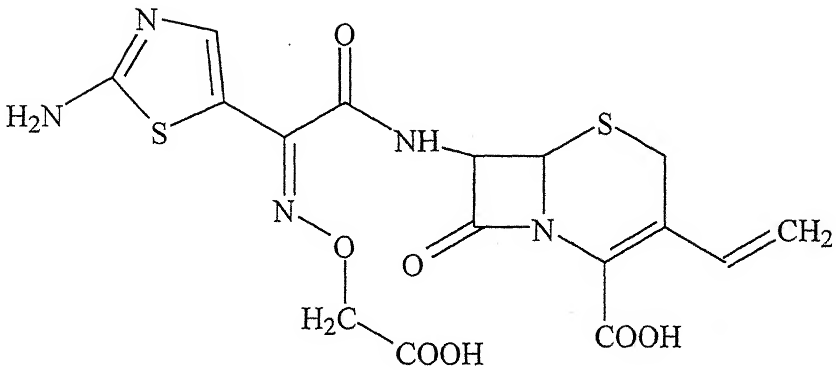
One can crosscheck by following two ways whether developed method is also stability indicating or not.

1. To carry out forced degradation studies using following conditions, and check main component is contaminated by other impurities or not using Photo Diode Array detector(i.e. by Spectrum correlation and contour plot)

- Acidic condition
- Alkaline condition
- Thermal condition
- Photochemical condition(By UV radiation)
- Oxidative condition, and
- Mobile phase condition

2.To isolate impurities from main compound and check resolution.
We have considered both the parameters and validated assay method of Cefixime and Cefdinir.

CEFIXIME



CEFIXIME

Chemical Names :

- [6R-[6α,7β(Z)]]-5-thia–1-azabicyclo[4.2.0]oct–2-ene-2-carboxylic acid, 7-[[(2-amino-4-thiazolyl)[(carboxymethoxy)imino]acetyl]-amino]-3-ethenyl-8-oxo,trihydrate.
- (6R,7R)-7-[2-(2-Amino-4-thiazolyl) glyoxylamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo [4.2.0]oct-2-ene-2-carboxylic acid, 7^2 –(Z)-[O-(carboxymethyl)oxime]trihydrate.
- [6R-[6α,7β(Z)]]-7-[[(2-Amino-4-thiazolyl)[(carboxymethoxy)-imino]acetyl]aminol-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

Nonproprietary name : Cefixime trihydrate

Proprietary Names : Oroken, Cefspan, Suprax

Empirical Formulae :

- Free acid $C_{16}H_{15}N_5O_7S_2$
- Trihydrate $C_{16}H_{15}N_5O_7S_2 . 3H_2O$

Molecular Weight :

- Free acid 453.44 [$C_{16}H_{15}N_5O_7S_2$]
- Trihydrate 507.49 [$C_{16}H_{15}N_5O_7S_2 . 3H_2O$]

CAS Number : 79350 – 37 –1

Appearance : The Z-isomer of cefixime, which is the article of commerce used in dosage form, is a white to light-yellow crystalline powder. It is odorless, or can have a slight characteristic odor.
The E-isomer of cefixime is a pale yellow solid.

Use and Applications : Cefixime is the first member of what is generally termed the third generation orally active cephalosporins. These third generation cephalosporins are distinct from the older β-lactam antibiotics in their intensive antibacterial activity against a wide range of gram-negative bacteria. The exceptional antibacterial activity of the third generation cephalosporins has been shown to be based on both their enhanced affinity for the target enzymes and their high stability to β-lactamase[1]. That

Cefixime shares similar characteristics with other third-generation cephalosporins was confirmed by Yasutaka Shigi et al.[2]. Although Cefixime is less active against staphylococci than are other orally active β-lactam antibiotics, it is far more potent against a wide range of gram-negative bacteria.

The aminothiazole ring appears to be associated with both excellent activity and oral adsorption, and the amino group in the thiazole ring is essential for the potential antibacterial activity[3]. Cefixime exhibits geometrical isomerism with reference to the configuration of oxime. The Z-isomer is the predominant isomer relative to the E-isomer, and is the article of commerce used in the preparation of dosage forms. The E-isomer is considered as the concomitant component of Cefixime, and is therefore not considered as an impurity in the usual pharmacopoeial sense. The antimicrobial activity and oral absorbability of both isomers have been studied in detail. The E-isomer is reported to be 2-32 times less active than the Z-isomer against gram-negative bacteria, although both isomers show appreciable oral absorbability regardless of the configuration of the oxime. In the marked contrast to other cephalosporin antibiotics, Cefixime has a vinyl group at C-3 and a (Z)-2-(2-amino-4-thiazolyl)-2-(carboxymethoxyimino)acetyl moiety at C-7 which influences its improved activity against gram-negative bacteria and pharmacokinetic properties[4,5].

Methods of Preparation : Excellent and efficient route for the synthesis of Z-isomer and E-isomer of cefixime have been developed[3,6,7]. The synthetic routes for the Z-isomer are shown in Scheme 1 and Scheme 2.

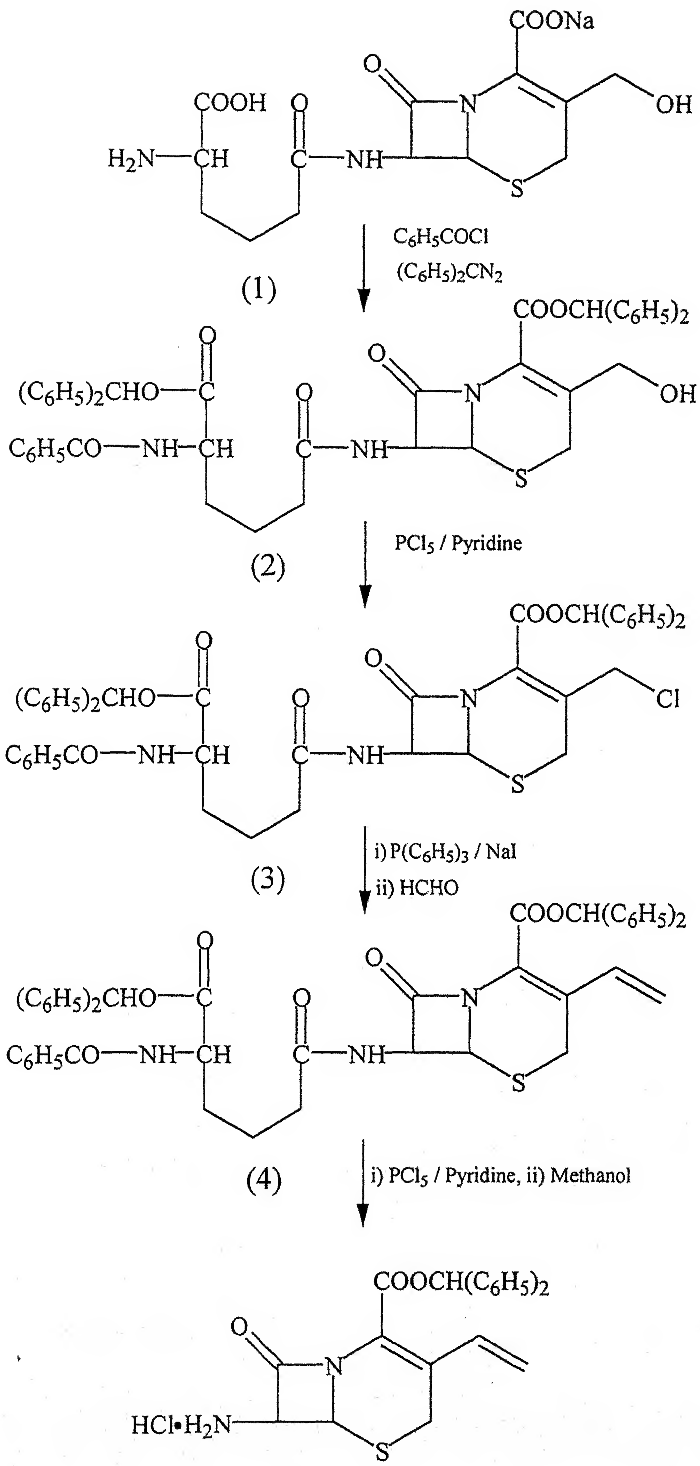
The synthesis involves the preparation of the key intermediate diphenylmethyl 7-amino-3-vinyl-3-cephem-4-carboxylate hydrochloride(5), and three different synthetic routes for the preparation of (5) have been developed and described by Kawabata et al.[7]. The more efficient of the three route described in Scheme 1, which starts with deacetylcephalosporin C sodium salt (1). This compound is treated with benzoyl chloride, followed by reaction with diphenyldiazomethane, to obtain the protected deacetylcephalosporin C (2). The reaction of (2) with phosphorus pentachloride and pyridine converts the hydroxymethyl group into a chloromethyl group, to yield intermediate (3). The chloromethyl derivative (3) is then treated with triphenylphosphine and sodium iodide in N,N-dimethyl-formamide to yield a phosphonium salt, which then treated with formaldehyde in methylene chloride (Wittig reaction) to obtain the 3-vinylderivative (4). Cleavage of the acyl side chain in intermediate (4) is achieved by treatment with phosphorus pentachloride and pyridine, followed by methanol, to give the intermediate (5) in good yield.

As shown in Scheme 2, the conversion of intermediate (5) into the Z-isomer of cefixime has been achieved by two different routes. In route A,the Intermediate (6),(Z)-2-(2-formamido-4-thiazolyl)-2-(tert-butoxycarbonylmethoxyimino) acetic acid, is initially activated by treating with a mixture of phosphoryl chloride and dimethylformamide (Vilsmeir reagent). This is condensed with intermediate (5) to give the protected intermediate (7). Deprotection of the N-formyl group in (7) is achieved by treatment with a methanolic solution of concentrated hydrochloric acid, obtaining the deformyl cephem intermediate (8). The removal of the tert-butyle and diphenylmethyl groups in (8) is achieved by treatment with trifluoroacetic acid and anisole to yield cefixime(9).

In the alternate synthetic route B, the intermediate (5) is acylated with 4-chloro-2-methoxyimino-3-oxobutyric acid (10) to give theacylated cephem intermediate (11). This compound is then treated with thiourea to give intermediate (12). 2-aminothiazol cephem. The cleavage of the diphenylmethyl ester of (12) is achieved by treatment with trifluoroacetic acid and anisole to give the monoester, (13), which on hydrolysis with sodium bicarbonate yields cefixime.

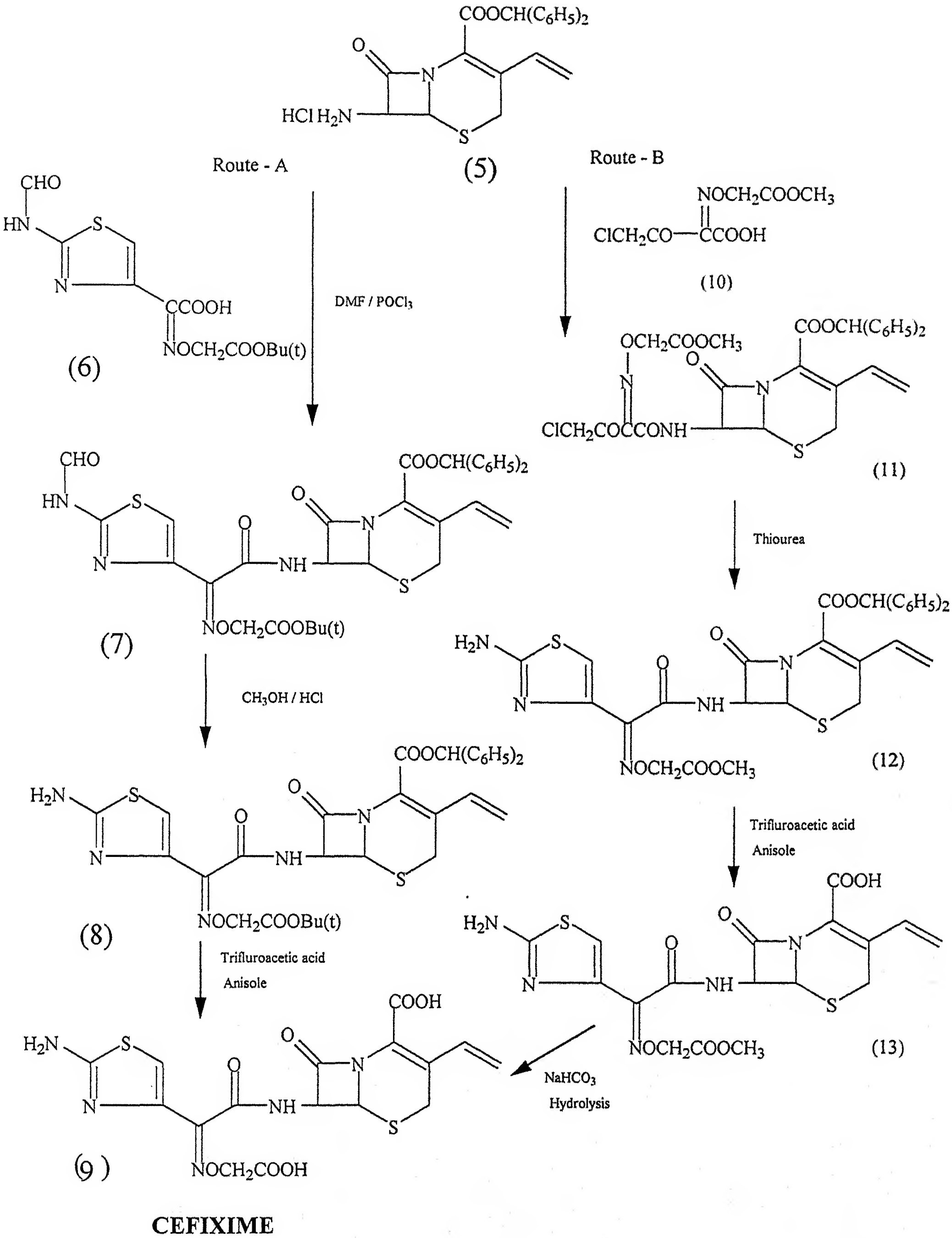
During their efforts to investigate the effect of oxime configuration in the 7-acyl side chain on the antibacterial activity and oral absorbability in rats, Kawabata et al. Synthesized the E-isomer of cefixime by two different methods[6]. In one of their methods, shown in Scheme 3 and 4, the key intermediate, 4-chloro-2-nitrobenzyloxycarbonylmethoxyimino-3-oxobutryic acid (17) is obtained as a mixture of geometrical isomers starting from ter-butyl (Z)-2-hydroxyimino-3-oxobutyrate (14). The geometrical isomers, 17-Z and 17-E, are indistinguishable in their spectral characteristics, and were characterized by separately converting them to the cyclic aminothiazole compounds, 18-Z and 18-E.

The E intermediate is activated with Vilsmeir reagent, and condensed with (5) to give the acylated compound (19). Intermediate (19) is then cyclized with thiourea to yield the aminothiazole cephem, (20). The diphenylmethyl group in (20) is removed by treatment with trifluoroacetic acid and anisole, and then subjected to alkaline hydrolysis to yield the E-isomer of cefixime.

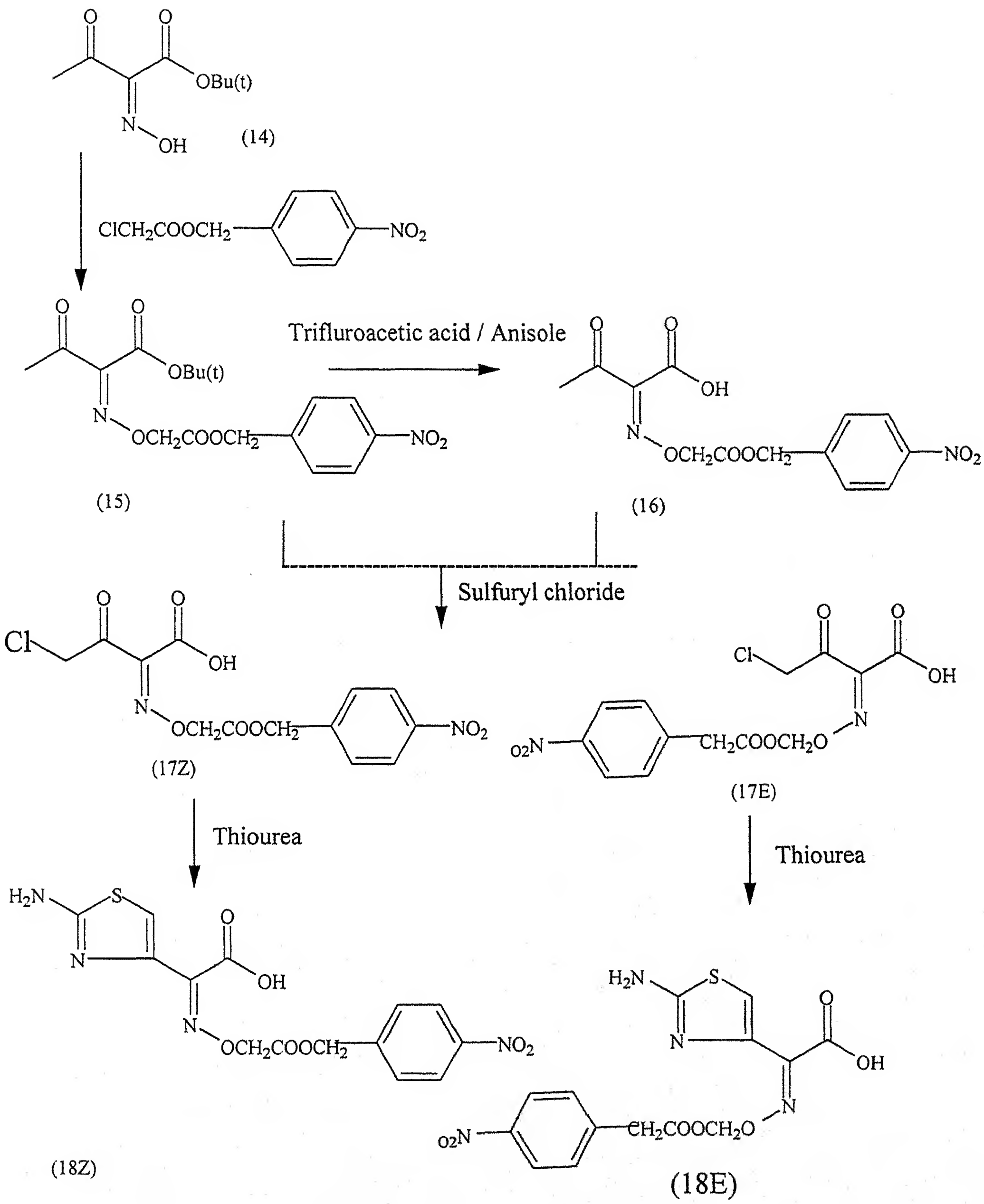


(5)

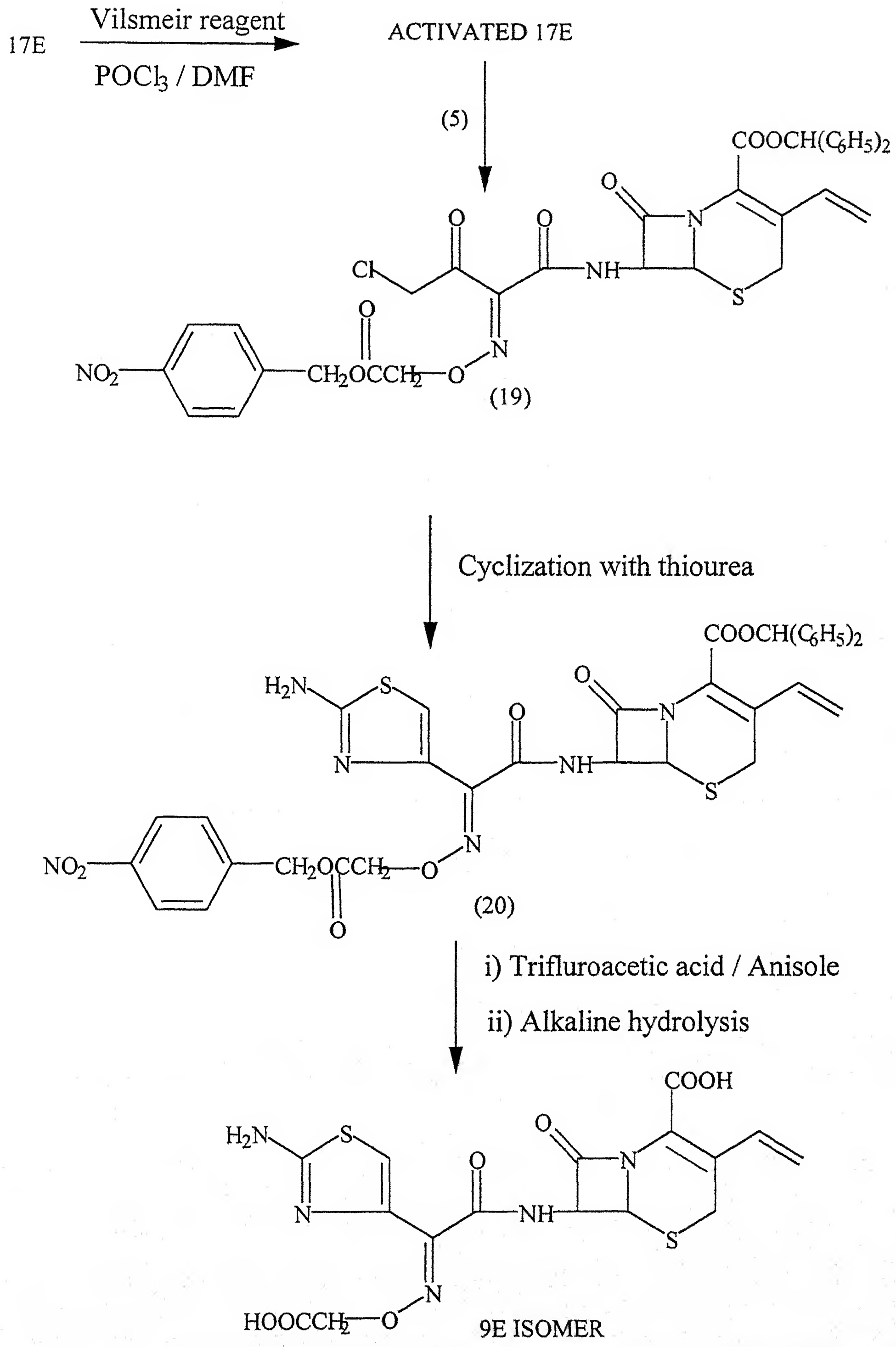
Synthetic route for the preparation of intermediate 5.



Synthetic route from intermediate 5 for the preparation of the Z- isomer of cefixime



OBu(t)
OH
(14)
ClCH2COOCH2
NO2
OBu(t)
OCH2COOCH2
NO2
(15)
Trifluroacetic acid / Anisole
OH
OCH2COOCH2
NO2
(16)
Sulfuryl chloride
Cl
OH
OCH2COOCH2
NO2
(17Z)
Cl
OH
O2N
CH2COOCH2O
(17E)
Thiourea
Thiourea
H2N
OH
OCH2COOCH2
NO2
(18Z)
H2N
OH
O2N
CH2COOCH2O
(18E)



Synthetic route for preparation of the E- isomer of cefixime

Physical Properties : This property can be checked by Scanning electron photomicrograph and by X-ray powder diffraction pattern.

Optical Rotation : The specific rotation of cefixime at 25°C in 10mg/ml aqueous solution of sodium bicarbonate is between –75° and –88°.

Thermal Analysis : This analysis can be carried out by Melting point apparatus, Differential scanning calorimeter, and Thermogravimetric analysis.

Hygroscopicity : When exposed to relative humidities ranging from 23% to 93% (at an equilibrium temperature of 25°C), cefixime trihydrate did not exhibit any measurable moisture pickup. The trihydrate phase is therefore determined to be non-hygroscopic.

Solubility Characteristics : Freely soluble in methanol, soluble in acetone and in glycerin, slightly soluble in alcohol, very slightly soluble in 70% sorbitol and practically insoluble in ether,in ethylacetate, and water.

Spectroscopy

1. **Vibrational Spectroscopy** : The infrared absorption spectra of Z- and E-isomers of cefixime were obtained using Perkin-Elmer 1650 FT-IR spectrophotometer, where the KBr pellet method of sample preparation was used to prepare the samples.As would be expected, the two isomers

may be differentiated on the basis of their absorption bands in the C=N oxime region.

Assignments for the I.R. of Cefixime Trihydrate

Energy (cm^{-1})	Band Assignment
3566,3531	NH stretch of the hydrogen-bonded amide group.
3295	Symmetric and antisymmetric NH stretches of the carbamate NH2 group.
2946	6-H,7-H stetching modes in the β-lactam ring
2946	CH3 asymmetric stretching mode
1772	β-lactam C=O stretching mode
1735	Carbamate C=O stretching mode
1670	Amide I C=O stretching mode
1591	Oxime C=N stretching mode
1457	CH3 deformation in the CH3O group
1336	Carbamate NH2 bending mode
1095	C-O stretch in the CH3O group
1063, 1025	C-O and N-O stretches of the carbamate and oxime moities in the CH2O groups

2. **Nuclear Magnetic Resonance Spectrometry** : ^{1}H – Spectrum :The ^{1}H-NMR spectra of the Z- and E-isomers of Cefixime were obtained using 200 MHz Bruker Instrument model DRX-200 NMR spectrometer. The data were obtained at ambient temprature in DMSO-d6, at a concentration of 10

mg/ml. The nomenclature of the assignments is based on the following alphabetical designation.

Chemical Shift	**Multiplicity**	**Relative number of protons**
9.6	Doublet	1
7.28	Broad	2
6.93 – 6.98	Multiplet	1
6.72	Singlet	1
5.78 – 5.84	Quartet	1
5.55	Doublet	1
5.29	Doublet	1
5.22	Doublet	1
4.60	Singlet	2
3.53	Quartet	2

3. **Mass Spectrmetry** : The mass spectra of Z- and E-isomers of cefixime were obtained using a PE Biosystem API-3000 instrument, and are shown in figure. The spectrum of cefixime is characterized by the presence of an intense protonated moleculer ion at m/z 454 (MH^+).

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8. Analytical Profiles of Drug Substances and Excipients vol.25.

Validation of Cefixime Trihydrate Assay Method

VALIDATION CRITERIA

	TEST PERFORMED	LIMITS
1.	**Specificity**	Resolution not less than 2.0 Tailing factor 2.0 (max.) Theoretical plate not less than 4500
2.	**Accuracy**	% Recovery 100 ± 1.5 %
3.	**Precision**	
	(I) Repeatability	RSD of Area & RT NMT 1.0%
	(II) Intermediate Precision	RSD of Area & RT NMT 1.0%
	(III) Reproducibility	Analytical error allowed up to 1.0%
4.	**Linearity**	Coefficient of correlation = 0.999
5.	**Robustness**	Resolution not less than 2.0 Tailing factor 2.0 (max.) Theoretical plate not less than 4500
6.	**Degradation study**	Cefixime peak should not be contaminated by other impurity
7.	**Solution stability**	Peak area difference from initial value should be not less than 1.0%

CEFIXIME

Method Validation For The Determination Of Assay

OBJECTIVE: To ensure the validity of analytical procedure for the determination of Assay.

SCOPE: The analytical method is validated as per ICH Guidelines Q2 A & B for the following parameters :-

1. Specificity
2. Linearity
3. Accuracy
4. Precision
5. Robustness
6. Degradation study
7. Solution Stability

HPLC CONDITIONS FOR ASSAY

Preparation of Mobile Phase :

25.0ml of Tetrabutylammonium hydroxide (40.0% in water) dissolved in 1000 ml distilled water. Add 370.0ml acetonitrile and adjust the pH 6.5 by H_3PO_4

Solvent: (For Dilution Purpose) Mobile phase was used for dilution purpose.

Name of Equipment

Instrument	Shimadzu LC-10AVP series
System No.	15,16,and 22
HPLC Pump	Shimadzu LC-10ATVP
HPLC Detector	Shimadzu SPD–M10AVP&SPD-10AVP
Auto Injector	SIL-10ATVP
System Controller	SCL-10AVP
Column Oven	CTO-10ASVP
Integrator	Computer P-III with Class-VP software ver 5.03
Balance	03

Chromatographic Parameters

Column	Inertsil ODS-3V (4.6 X 150mm), 5µ S.No. OEI83044
Mobile Phase	2.5% Tetra butyl ammonium hydroxide(40% in water) : Acetonotrile
Composition	73 :27
pH	6.5 by H_3PO_4
Flow Rate	1.0 ml / min.
Wavelength	254 nm
Column Oven Temp.	40°C
Aux. Range	2.0
Injection Volume	20 µl

SPECIFICITY OF CEFIXIME

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

Standard preparation:- Weigh accurately 0.05 gm of sample B.No.019/SR/VII/369/39 in 100 ml volumetric flask, dissolved and diluted to the mark with distilled water & heated at 80°c for 1hr to get E-Isomer for resolution .

Observation

S.No	Sample Name	File Name	R.T.	Theoretical Plates	Tailing Factor	Resolution
1.	E-Isomer	FE0610.01	18.54	9391.78	1.09	
2.	Cefixime	FE0610.01	21.4	6741.65	1.69	3.16

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-Isomer	5000	2.0	
Cefixime	4500	2.0	2.0

Conclusion:

- Theoritical plate ,Tailing Factor and Resolution were within the acceptable limit.

RECOVERY STUDY OF CEFIXIME

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found.

Stock Solution(A) – Weighed 0.0503 gm of Cefixime Batch No. 019/SR/VII/369/39 in 100 ml volumetric flask & diluted to the mark with Mobile phase.

Stock Solution(B) – Weighed 0.0547 gm of Cefixime Batch no. 019/DRR/VII/Demo/D1 in 50 ml volumetric flask & diluted to the mark with Mobile phase.

100.6 PPM - Pipetted 5ml of A solution + 0 ml of B in 25ml volumetric flask & diluted to the mark with Mobile phase.

218.8 PPM - Pipetted 0ml of A solution + 5 ml of B in 25ml volumetric flask & diluted to the mark with Mobile phase.

260.3 PPM - Pipetted 15ml of A solution + 5 ml of B in 50ml volumetric flask & diluted to the mark with Mobile phase.

520.6 PPM - Pipetted 15ml of A solution + 5 ml of B in 25ml volumetric flask & diluted to the mark with Mobile phase.

739.4 PPM - 15ml of A solution + 10 ml of B in 25ml volumetric flask & diluted to the mark with Mobile phase.

S.No.	Conc.	Theoretical Area	Practical Area	Recovery	File Name
1.	100.6	--	1832412.66	--	FE0710-Rep1-3.001
2.	218.8	--	3915376.33	--	FE0710-Rep1-3.002
3.	260.3	4706307.155	4700166.33	99.86%	FE0710-Rep1-3.003
4.	520.6	9412614.31	9394259.33	99.80%	FE0710-Rep1-3.004
5.	739.4	13327990.64	13269780.5	99.56%	FE0710-Rep1-3.005

**All above solutions were injected for three times to calculate % Recovery.

Average recovery - 99.74%

Remark - Average recovery were found within the acceptable limit.

PRECISION OF CEFIXIME

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the specified conditions.

Following determinations are carried out for establishing the Precision of Assay method.

- ➢ Repeatability
- ➢ Intermediate Precision
- ➢ Reproducibility

Repeatability

Sample preparation : Weighed 0.1255gm of Cefixime B.No.019/SR/VII/369/39 in 100 ml volumetric flask, dissolved and diluted to the mark with mobile phase. Solution concentration was 1255 PPM.

$$SD = \sqrt{\frac{\sum_{I=1}^{N} (xi - x)^2}{N - 1}}$$

$$\text{Relative Standard Deviation} = \frac{(\text{Standard Deviation} \times 100)}{\text{Average } (X)}$$

251 PPM:- Pipetted 10ml of 1255 PPM solution to 50ml volumetric flask & diluted to the mark with Mobile phase.

502 PPM :- Pipetted 10ml of 1255 PPM solution to 25ml volumetric flask & diluted to the mark with Mobile phase.

753 PPM :-Pipetted 15ml of 1255 PPM solution to 25 ml volumetric flask & diluted to the mark with Mobile phase.

S. No.	Conc. (ppm)	Avg. Area .	RSD of Area %	Criteria	RSD of RT %	Criteria	File Name
1.	251	4570918.16	0.068	RSD of Area = NMT 1.0%	0.15	RSD of RT = NMT 1.0%	FE0610-Rep1-6.03
2.	502	9116951.8	0.07		0.16		FE0610-Rep1-6.04
3.	753	13735899.16	0.161		0.023		FE0610-Rep1-6.05

**All the three solutions were injected for six times for calculating RSD of RT& Area.

Conclusion : RSD of Retention time & Area were found within the acceptable limit.

Intermediate Precision

A matrix of experiment is designed to include random circumstantial events on an analytical method.

Following changes are made.

- Analyst
- Instrument
- Inter day variation

Sample preparation : Weighed 0.0504gm of Cefixime B.No.019/SR/VII/369/39 in 100ml volumetric flask, dissolved and diluted to the mark with mobile phase. Solution concentration was 504ppm.

Sample preparation : Weighed 0.0504gm of Cefixime B.No. 019/SR/VII/369/39 in 100ml volumetric flask, dissolved and diluted to the mark with mobile phase. Solution concentration was 504ppm.

Sample preparation : Weighed 0.0497gm of Cefixime B.No. 019/SR/VII/369/39 in 100ml volumetric flask, dissolved and diluted to the mark with mobile phase. Solution concentration was 497ppm.

$$\mathbf{SD} = \sqrt{\frac{\sum_{I=1}^{N} (xi - x)^2}{N-1}}$$

$$\text{Relative Standard Deviation} = \frac{(\text{Standard Deviation}) \times 100}{\text{Average}(X)}$$

S.No.	Conc. (ppm)	Avg. Area	RSD of Area	Criteria	RSD of RT	Criteria	Analyst
1.	504	9089153.66	0.27%	1.0%	0.086%	1.0%	Analyst-1
2.	504	9153273.16	0.26%	1.0%	0.059%	1.0%	Analyst-2
3.	497	18273552.16	0.097%	1.0%	0.21%	1.0%	Analyst-3

**All above solutions were injected for six times to calculate the RSD of RT & Area.

Conclusion : RSD of Retention time & Area were found within the acceptable limit.

Reproducibility

To check the variation in assay of Cefixime using different systems and different analysts .

Standard preparation:-Weighed accurately about 0.05gm of standard sample B.No. 019/SR/VII/369/39 in 100ml volumetric flask, dissolved and diluted to the mark with mobile phase.

Test preparation :- Weighed accurately about 0.05gm of test sample in 100ml volumetric flask, dissolved and diluted to the mark with mobile phase.

Calculation :-

$$\text{\% Test Purity} = \frac{\text{Area of test}}{\text{Area of standard}} \times \frac{\text{Weight of standard}}{\text{Weight of test}} \times \text{Purity of standard}$$

Calculated Assays :

Batch No. : 019/DRR/VII/DEMO/D1

Analyst	Analysed on	System Used	As is Purity (%)	Max. Variation	Criteria
Analyst-1	28th OCT2000	SYSTEM No.15	88.55		
Analyst-2	20th OCT 2000	SYSTEM No.15	88.21		
Analyst-3	17th OCT 2000	SYSTEM No.16	88.00	0.55%	NMT1.0%

Batch No. : 019/DRR/VII/DEMO/D2

Analyst	Analysed on	System Used	As is Purity (%)	Max. Variation	Criteria
Analyst-1	28th OCT2000	SYSTEM No.15	88.38		
Analyst-2	20th OCT 200	SYSTEM No.15	88.22		
Analyst-3	17th OCT 200	SYSTEM No.16	87.93	0.45%	NMT 1.0

Batch No. : 019/SR/VII/337/27

Analyst	Analysed on	System Used	As is Purity (%)	Max. Variation	Criteria
Analyst-1	28th OCT2000	SYSTEM No.15	88.77		
Analyst-2	20th OCT 200	SYSTEM No.15	88.60		
Analyst-3	17th OCT 200	SYSTEM No.16	88.93	0.33%	NMT 1.0

Conclusion : Variation in assay from analyst to analyst and system to system were found within acceptable limit.

LINEARITY OF CEFIXIME

The linearity of an analytical procedure is its ability (within a given range) to obtain the test results which are directly proportional to the concentration of an analyte in the sample.

Solution Preparations :-

Stock solution-1:- 0.1251gm of Cefixime B.No. 019/SR/VII/369/39 was dissolved & diluted to 100ml volumetric flask to the mark with mobile phase.

The concentration of this solution was 1251 ppm.

250.2 PPM:-5ml of 1251 PPM solution was diluted to 25ml volumetric flask to the mark with Mobile phase.

375.3 PPM:-5ml of 750.6 PPM solution was diluted to 10ml volumetric flask to the mark with Mobile phase.

450.36 PPM:-15ml of 750.6 PPM solution was diluted to 25 ml volumetric flask to the mark with Mobile phase.

500.4 PPM:-10ml of 1251 PPM solution was diluted to 25ml volumetric flask to the mark with Mobile phase.

625.5 PPM:-5ml of 1251 PPM solution was diluted to 10 ml volumetric flask to the mark with Mobile phase.

750.6 PPM:-15ml of 1251 PPM solution was diluted to 25ml volumetric flask to the mark with Mobile phase.

S.No.	Concentration	Average Area	File Name
1.	250.2	4544344.6	FE1010-Rep1-3.03
2.	375.3	6780654.66	FE1010-Rep1-3.04
3.	450.36	8229462.66	FE1010-Rep1-3.05
4.	500.4	9066123	FE1010-Rep1-3.06
5.	625.5	11255127.66	FE1010-Rep1-3.07
6.	750.6	13704776.66	FE1010-Rep1-3.08

Observation :

Component Name	Coefficient of correlation	Criteria
CEFIXIME	**0.9999**	**NLT 0.999**

Conclusion :

- Graph plotted Area v/s Concentration of Cefixime was found linear for the range 250.2 to 750.6ppm.
- Coefficient of correlation is also within the acceptable range.

ROBUSTNESS OF CEFIXIME

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during normal usage.

Typical variations are :-

- Influence of variations in column oven temperature.
- Influence of variations on flow rate.
- Influence of variations in buffer strength.
- Influence of variations of pH in mobile phase.
- Influence of variations in mobile phase composition.

Sample preparation:-

Weigh accurately 0.05gm of sample B.No. 019/SR/VII/369/39 in 100ml volumetric flask diluted to the mark with distilled water. Heated the above solution in water bath for 1hr at 80°c to get the E-Isomer for resolution. This solution is used for entire Robustness study.

HPLC CONDITION :-

Column	**INERTSIL-ODS 5μ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	6.5 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.Time	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	18.42	8800.96	1.06		FE1210.01
Cefixime	21.28	6542.77	1.63	3.11	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Column Oven temperature by +10°c

HPLC CONDITION :-

Column	**INERTSIL-ODS 5μ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	6.5 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	50°C
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked .

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	17.59	9610.41	1.06		FE1210.03
Cefixime	20.16	6874.89	1.69	3.05	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion :Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Column Oven temperature by -10°c

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	6.5 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	30°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked .

Observation :

Sample Name	R.Time	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	18.79	8274.48	1.05		FE1210.02
Cefixime	21.88	6212.28	1.59	3.19	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Flow Rate by +20%

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	6.5 H_3PO_4
Flow	1.2 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.Time	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	15.12	8091.63	1.05		FE1210.04
Cefixime	17.46	6162.68	1.58	3.0	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Flow Rate by -20%

HPLC CONDITION :-

Column	**INERTSIL-ODS 5μ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	6.5 H_3PO_4
Flow	0.8 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	22.81	9910.15	1.07		FE1210.05
Cefixime	26.38	6847.6	1.68	3.25	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Strength of buffer salt in mobile phase by + 5%

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.625% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	6.5 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked .

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	16.96	8168.44	1.04		DC1410.06
Cefixime	19.50	6714.53	1.52	2.98	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Strength of buffer salt in mobile phase by -5.0%

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.375% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	6.5 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked .

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	15.37	6216.85			DC1410.04
Cefixime	18.25	4870.31	1.5	3.15	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed pH of mobile phase by + 0.5 units

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	73 : 27
pH	7.0 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked .

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	13.192	6545.68	1.04		DC1410.009
Cefixime	15.292	5647.61	1.34	2.86	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed pH of mobile phase by - 0.5 units

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	75 : 25
pH	6.0 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked .

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	24.13	8926.51	1.07		DC1410.02
Cefixime	27.89	5754.03	1.78	3.02	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resoluti on
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed Organic Mobile phase composition by - 2% i.e. Acetonitrile

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	75 : 25
pH	6.5 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	20.82	7581.57	1.04		DC1410.07A
Cefixime	24.367	5771.31	1.44	3.16	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2.0	2.0
Cefixime	4500	2.0	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed Organic Mobile phase composition by +2% i.e. Acetonitile

HPLC CONDITION :-

Column	**INERTSIL-ODS 5µ (4.6 x 150mm)N0. OE183051**
Mobile Phase	2.5% Tetra butyl ammonium hydroxide (40% in water) : Acetonitrile
Composition	71 : 29
pH	6.5 H_3PO_4
Flow	1.0 ml/ min.
Wavelength	254 nm
Column Oven Temp.	40°C
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked .

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
E-isomer	16.983	8119.64	1.06		DC1410.008
Cefixime	19.45	6051.46	1.56	2.81	

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
E-isomer	5000	2	
Cefixime	4500	2	2.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

DEGRADATION STUDY OF CEFIXIME

To establish the validity of assay method for Cefixime subjected to stress conditions of storage.

Experimental Condition : Degradation of Cefixime was checked under the following stressed storage conditions.

(a) Mobile Phase condition(at 25°c)

(b) Exposure to Photochemical condition (Solid & Solution)

(c) Acidic condition (1N Hydrochloric Acid)

(d) Alkaline condition (1N Sodium Hydroxide)

(e) Oxidative condition (8.2% Hydrogen Peroxide)

(f) Thermal condition (About 50°C in water bath)

Cefixime B.No. **019/SR/VII/369/39** was taken for the study purpose.

Following are the criteria for conclusion :

Spectrum: Correlate the spectrum of Cefixime initially & after the experimental condition. The spectrum correlation value more than 99% indicates that no other peak merge with Cefixime peak.

Contour Plot: Contour plots were taken out initially and at the end of experiment. Cefixime main peak having similar nucleus, indicates that Cefixime main peak is pure and not contaminated with any degraded product formed during stressed conditions.

Degradation study in Mobile Phase

To check the degradation of Cefixime in Mobile phase at 25°c.
Solution preparation :

(a) Stock solution :Weighed 0.2512gm of Cefixime (B.No. 019/SR/VII/369/39), dissolved and diluted in 100ml volumetric flask and make up to the mark with mobile phase.

(b) Initial solution : Pipetted out 5ml from stock solution immediately after preparation & diluted in 25ml volumetric flask to the mark with mobile phase.

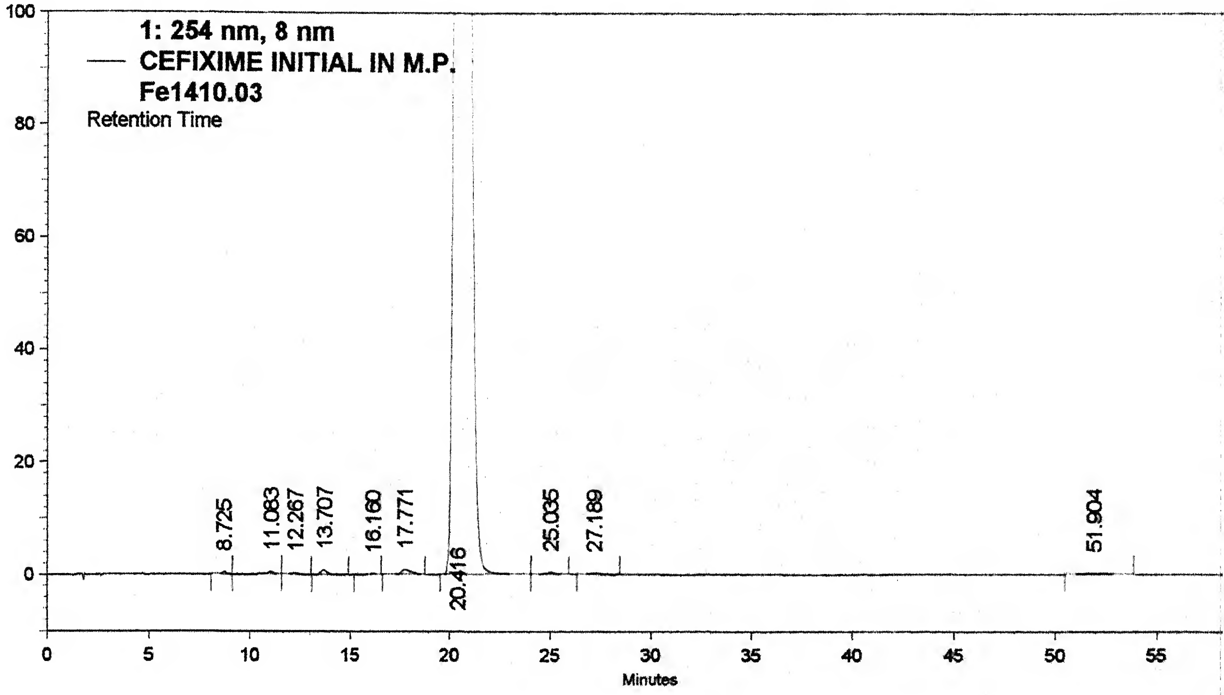
(c) Subsequent solutions: Pipetted out 5ml from stock solution & diluted in 25ml volumetric flask to the mark with mobile phase at two hour interval up to 8hrs, then 12hrs, 24 hrs and 48 hrs with mobile phase.

S.No.	Time	Area	% Variance	File Name
01.	Initial	18325234		FE1410.03
02.	After 2 Hrs.	18397462	0.39	FE1410.05
03.	After 4 Hrs.	18309504	0.085	FE1410.07
04.	After 6 Hrs.	18434951	0.59	FE1410.08
05.	After 8 Hrs.	18409184	0.46	FE1410.09
06.	After12 Hrs.	18269826	0.30	FE1410.10
07.	After 24 Hrs.	18070111	1.39	FE1510.06
08.	After 48 Hrs.	17919742	2.21	FE1610.01

Conclusion :

(1) Spectrum correlation of Cefixime peak at initial & after 48hrs shows correlation is 100%. Which confirms that no other peak is merging with Cefixime.
(2) Contour plot of Cefixime peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefixime.
(3) Percentage variance in area of cefixime from initial to 12 hrs was within the acceptable range.

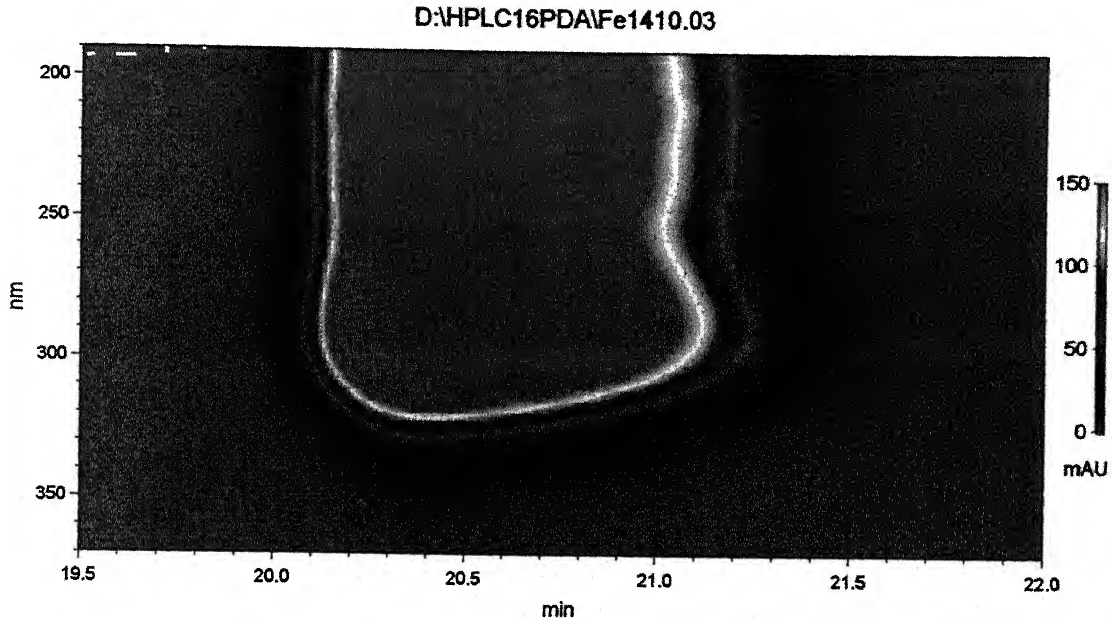
Method Name : **C:\CLASS-VP\Methods\CFXPDA.met**
File Name : **D:\HPLC16PDA\Fe1410.03**
Acquired Time : **10/14/00 11:43:36 AM**
Sample ID : **CEFIXIME INITIAL IN M.P.**



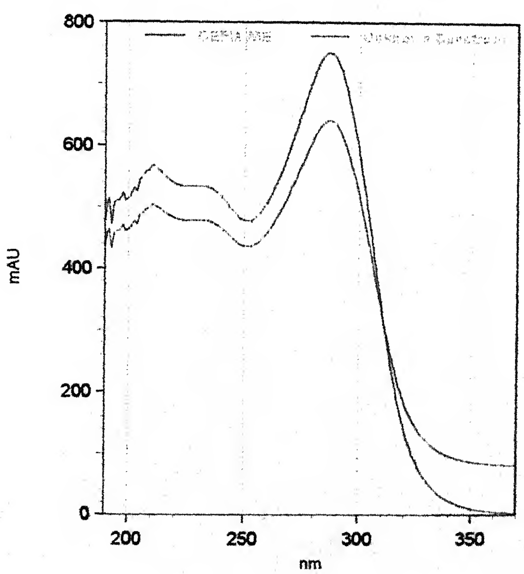
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	8.725	8262	0.04	
2	11.083	19452	0.11	
3	12.267	7629	0.04	
4	13.707	22039	0.12	
5	16.160	4542	0.02	
6	17.771	35874	0.19	
7	20.416	18325234	99.19	CEFIXIME
8	25.035	16698	0.09	
9	27.189	12360	0.07	
10	51.904	23143	0.13	
Totals		18475233	100.00	

Method Name : **C:\CLASS-VP\Methods\CFXPDA.met**
File Name : **D:\HPLC16PDA\Fe1410.03**
Acquired Time : **10/14/00 11:43:36 AM**
Sample ID : **CEFIXIME INITIAL IN M.P.**

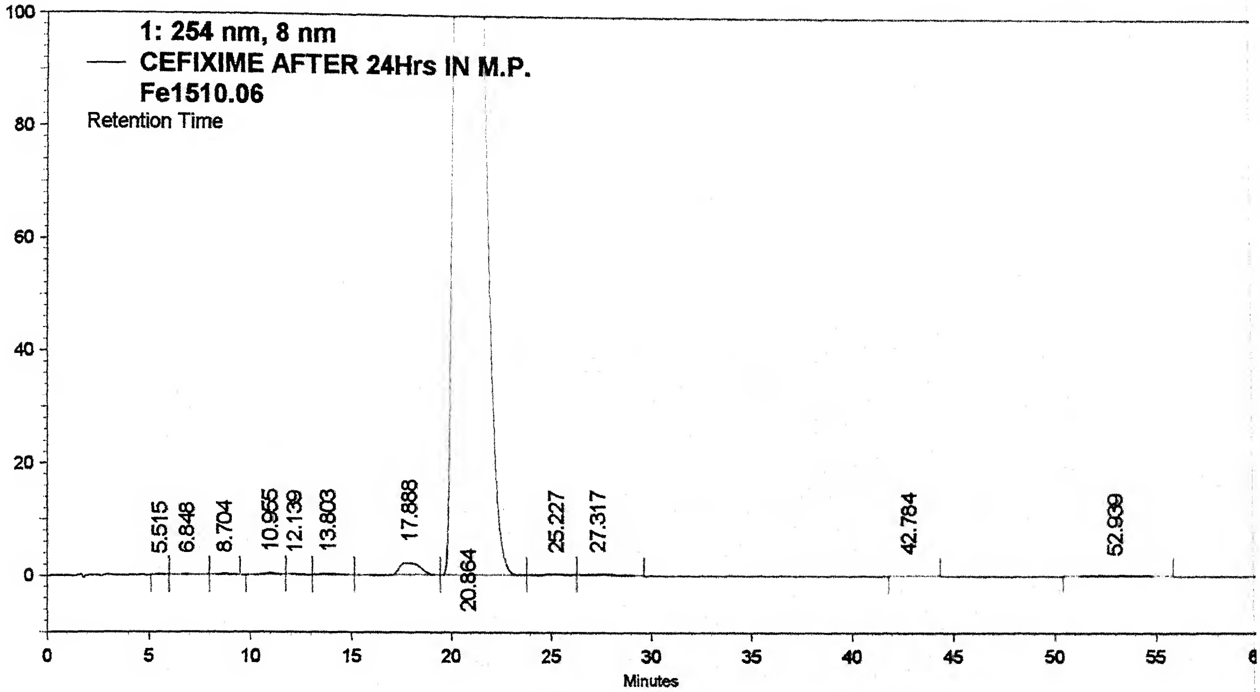


Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 1.000000
Library: C:\CLASS-VP\Spec\CFXmp.lib

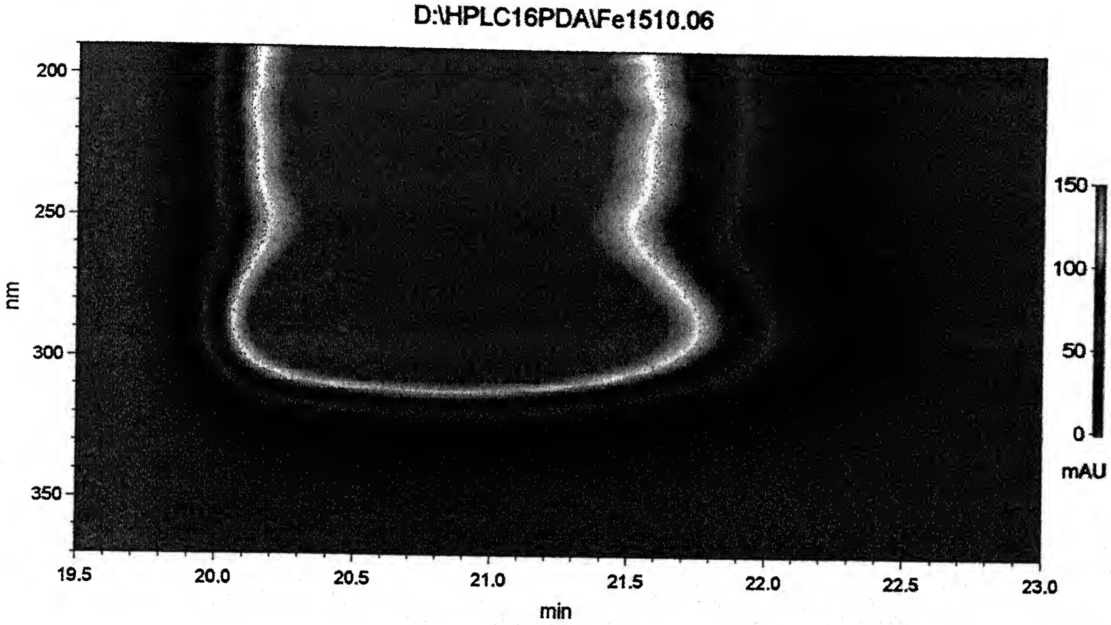
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.06
Acquired Time : 10/15/00 12:41:12 PM
Sample ID : CEFIXIME AFTER 24Hrs IN M.P.



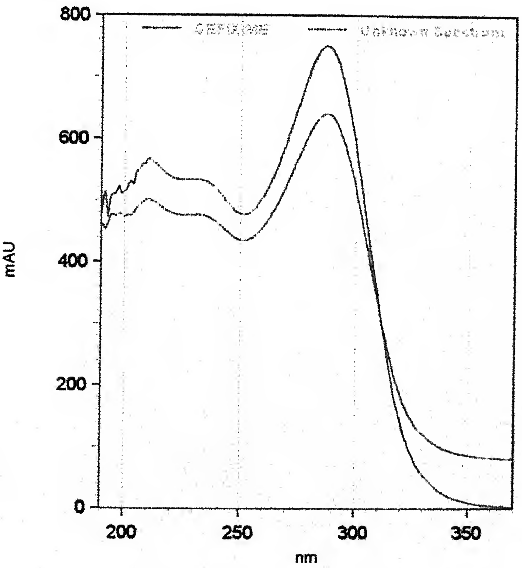
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	5.515	2781	0.02	
2	6.848	3069	0.02	
3	8.704	11903	0.06	
4	10.955	17344	0.09	
5	12.139	6099	0.03	
6	13.803	21508	0.12	
7	17.888	169265	0.92	
8	20.864	18070111	98.33	CEFIXIME
9	25.227	18221	0.10	
10	27.317	29702	0.16	
11	42.784	5412	0.03	
12	52.939	20661	0.11	
Totals		18376076	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.06
Acquired Time : 10/15/00 12:41:12 PM
Sample ID : CEFIXIME AFTER 24Hrs IN M.P.



Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999928
Library: C:\CLASS-VP\Spec\CFXmp.lib

Degradation study in Photochemical Condition-1

To check the degradation of Cefixime (B.No. 019/SR/VII/369/39) in Ultra Violet Radiation. The study carried out with solid state under UV light.

Experimental Condition: 1gm of Cefixime was taken into a open petri dish and kept under UV Radiation. Sample was analyzed before exposing to UV Radiation and after 12 hrs & 24 hrs of exposure to UV Radiation.

Initial Solution: Weighed 0.0507gm of Cefixime dissolved and diluted in 100ml volumetric flask and make up to the mark with mobile phase & injected immediately.

Sample Solution after 12 hrs: Weighed 0.0507gm of Cefixime from UV light exposed petridish dissolved and diluted in 100ml volumetric flask and make up to the mark with mobile phase & injected immediately.

Sample Solution after 24 hrs : Weighed 0.0506gm of Cefixime from UV light exposed petridish dissolved and diluted in 100ml volumetric flask and make up to the mark with mobile phase & injected immediately.

Solid, exposed in UV light :-

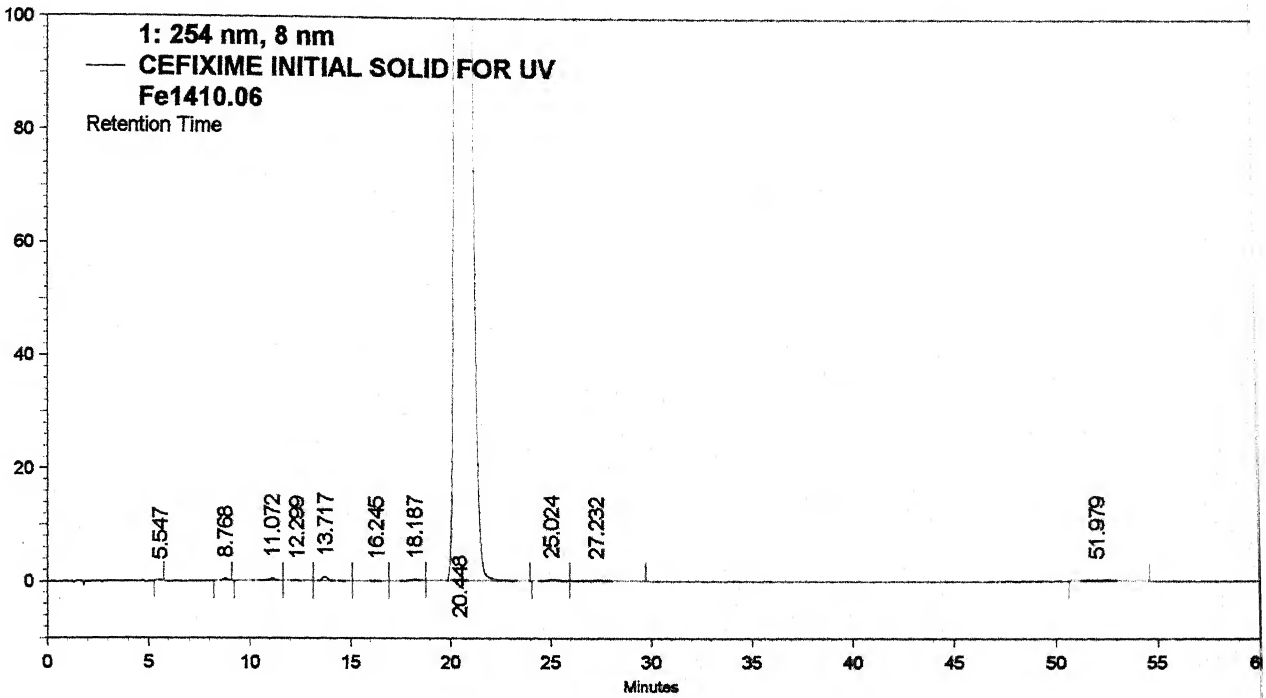
S.No.	Time	Area	% Variance	File Name
1.	Initial	18577278	-	FE1410.06
2.	After12Hrs.	18230322	1.86	FE1410.12
3.	After24Hrs.	18217922	1.93	FE1510.08

Conclusion :

(1) Spectrum correlation of Cefixime peak at initial & after 24.0hrs shows correlation is 100%. Which confirms that no other peak is merging with Cefixime.

(2) Contour plot of Cefixime peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefixime.

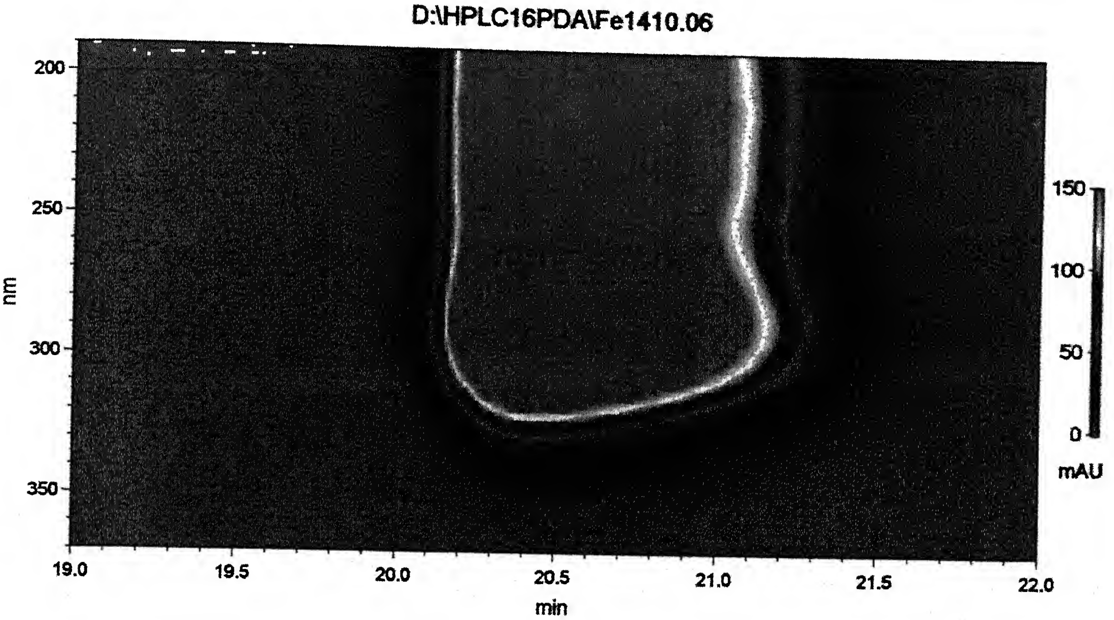
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1410.06
Acquired Time : 10/14/00 3:10:04 PM
Sample ID : CEFIXIME INITIAL SOLID FOR UV



1: 254 nm, 8 nm

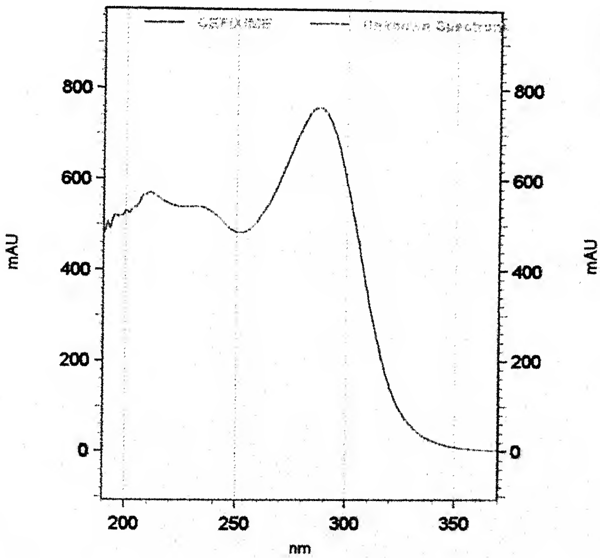
Pk #	Retention Time	Area	Area Percent	Name
1	5.547	1667	0.01	
2	8.768	7665	0.04	
3	11.072	17652	0.09	
4	12.299	5940	0.03	
5	13.717	21615	0.12	
6	16.245	4827	0.03	
7	18.187	13795	0.07	
8	20.448	18577278	99.34	CEFIXIME
9	25.024	12904	0.07	
10	27.232	11646	0.06	
11	51.979	25978	0.14	
Totals		18700967	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1410.06
Acquired Time : 10/14/00 3:10:04 PM
Sample ID : CEFIXIME INITIAL SOLID FOR UV



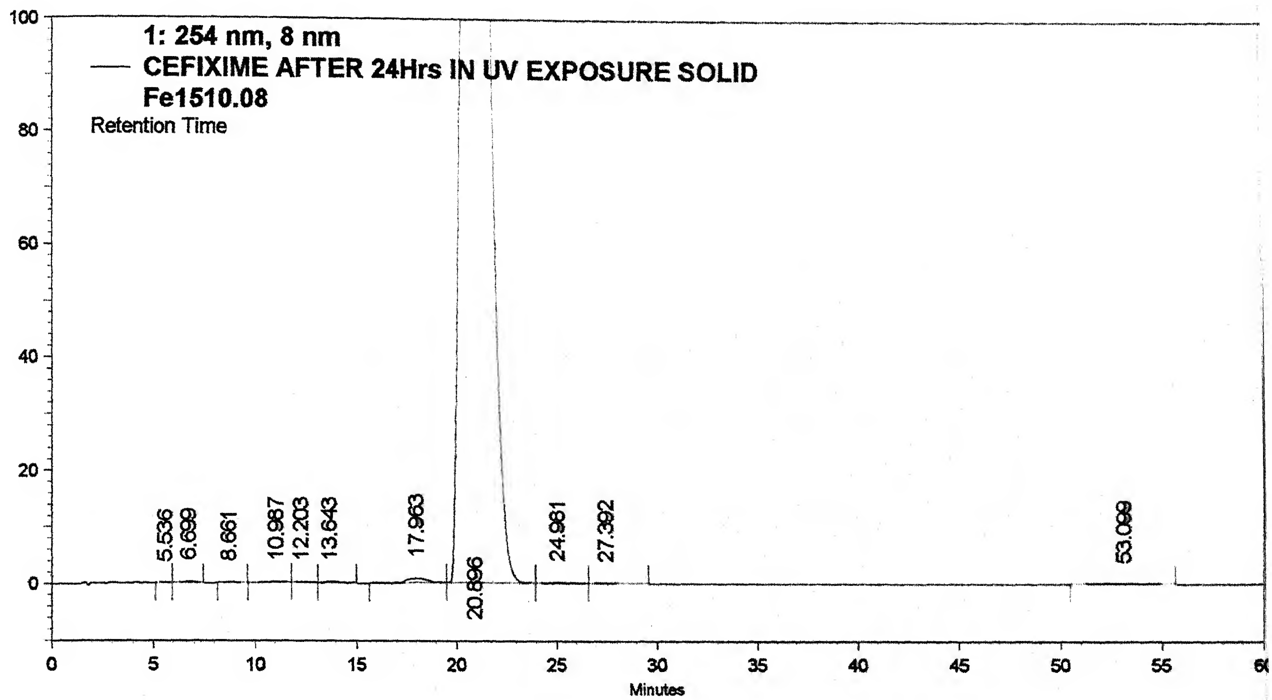
Peak Name: CEFIXIME

Number of Hits: 1



Hit 1
Name: CEFIXIME
Similarity Index: 1.000000
Library: C:\CLASS-VP\Spec\CFXuv1.lib

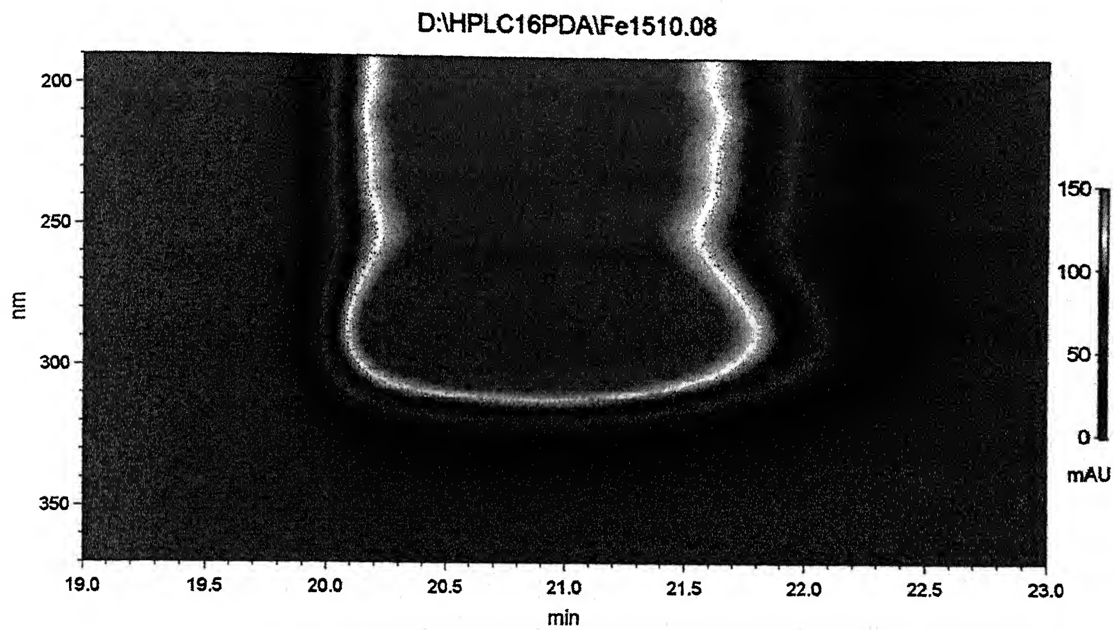
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.08
Acquired Time : 10/15/00 2:53:15 PM
Sample ID : CEFIXIME AFTER 24Hrs IN UV EXPOSURE SOLID



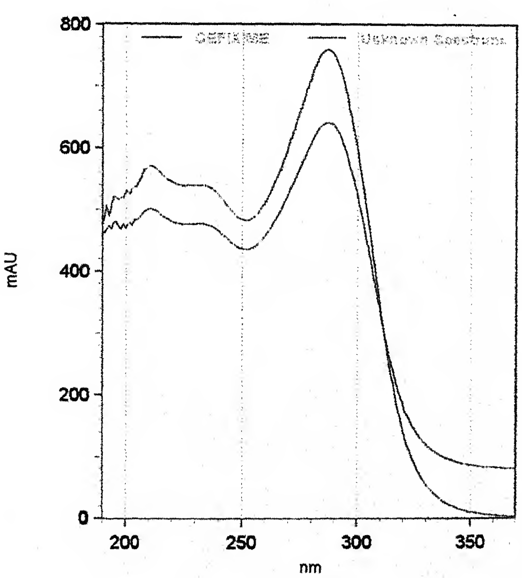
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	5.536	2170	0.01	
2	6.699	10697	0.06	
3	8.661	8220	0.04	
4	10.987	12576	0.07	
5	12.203	9408	0.05	
6	13.643	17373	0.09	
7	17.963	68161	0.37	
8	20.896	18217922	99.10	CEFIXIME
9	24.981	11580	0.06	
10	27.392	3891	0.02	
11	53.099	20798	0.11	
Totals		18382796	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.08
Acquired Time : 10/15/00 2:53:15 PM
Sample ID : CEFIXIME AFTER 24Hrs IN UV EXPOSURE SOLID



Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999914
Library: C:\CLASS-VP\Spec\CFXuv1.lib

Degradation study in Photochemical Condition -2

To check the degradation of Cefixime (B.No. 019/SR/VII/369/39) in Ultra Violet Radiation. The study carried out with solution state under UV light.

Experimental Condition : Weighed 0.051gm Cefixime in 100ml volumetric flask. Dissolved and diluted to the mark with mobile phase. Kept the flask under UV radiation for 24 hrs to expose .

Initial Solution: Injected the solution immediately after preparation.

Sample Solution after 12 hrs: Injected the solution kept under UV radiation after 12 hrs.

Sample Solution after 24 hrs : Injected the solution kept under UV radiation after 24 hrs.

Solution, exposed in UV light :-

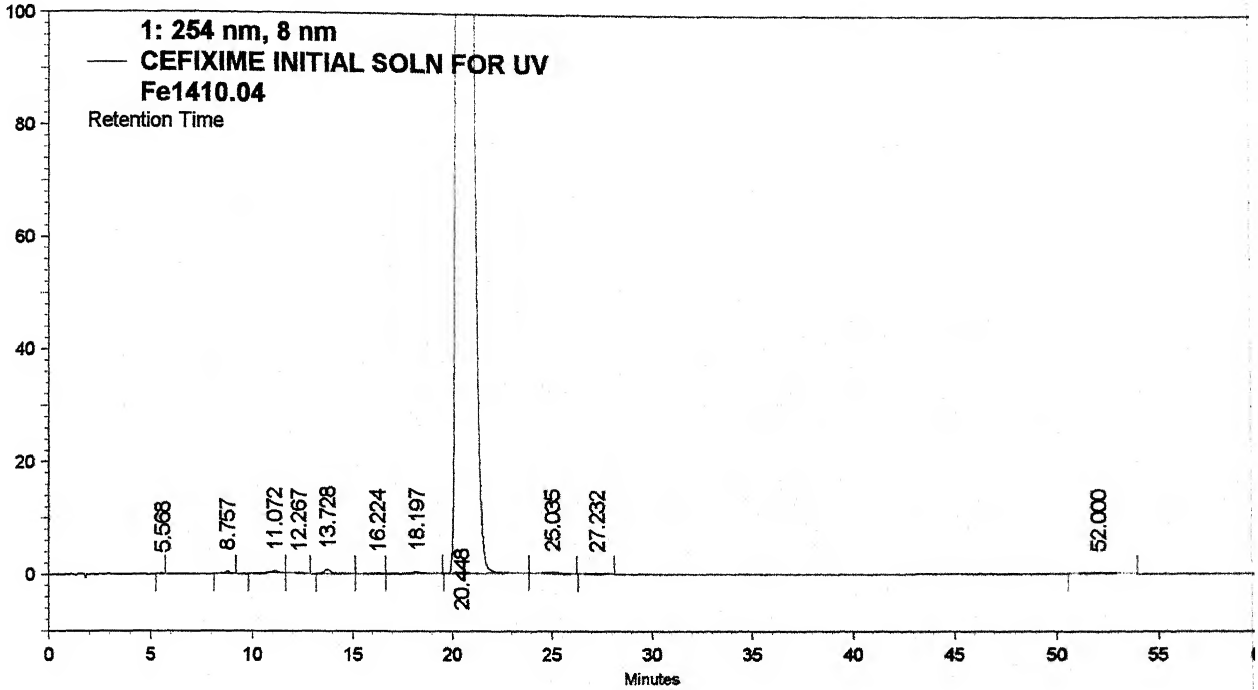
S.No.	Time	Area	% Variance	File Name
01.	Initial	18691566	-	FE1410.04
02.	After 12Hrs.	18646378	0.24	FE1410.11
03.	After24 Hrs.	18595671	0.51	FE1510.07

Conclusion :

(1) Spectrum correlation of Cefixime peak at initial & after 24.0hrs shows correlation is 100%. Which confirms that no other peak is merging with Cefixime.

(2) Contour plot of Cefixime peak from initial to final analysis showed same pattern confirming that no other peak is merging with the main peak of Cefixime.

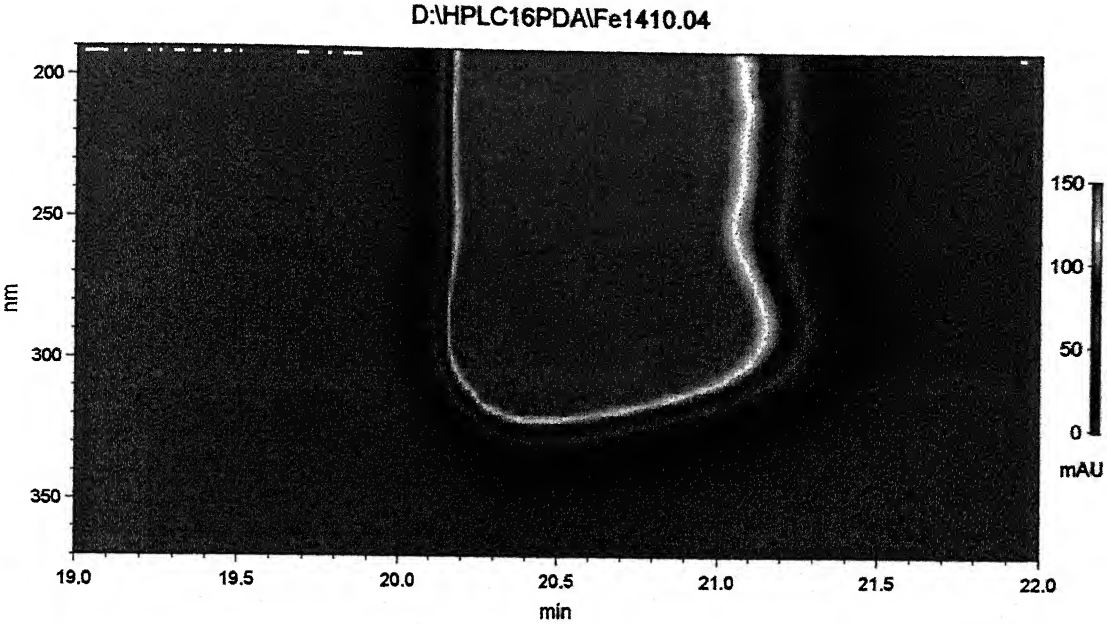
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1410.04
Acquired Time : 10/14/00 12:57:21 PM
Sample ID : CEFIXIME INITIAL SOLN FOR UV



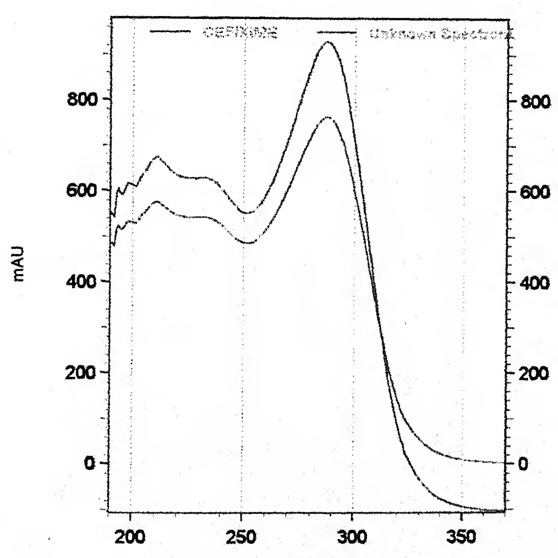
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	5.568	1644	0.01	
2	8.757	8167	0.04	
3	11.072	18182	0.10	
4	12.267	6068	0.03	
5	13.728	20527	0.11	
6	16.224	4577	0.02	
7	18.197	19039	0.10	
8	20.448	18695036	99.34	CEFIXIME
9	25.035	15350	0.08	
10	27.232	7311	0.04	
11	52.000	24133	0.13	
Totals		18820034	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1410.04
Acquired Time : 10/14/00 12:57:21 PM
Sample ID : CEFIXIME INITIAL SOLN FOR UV

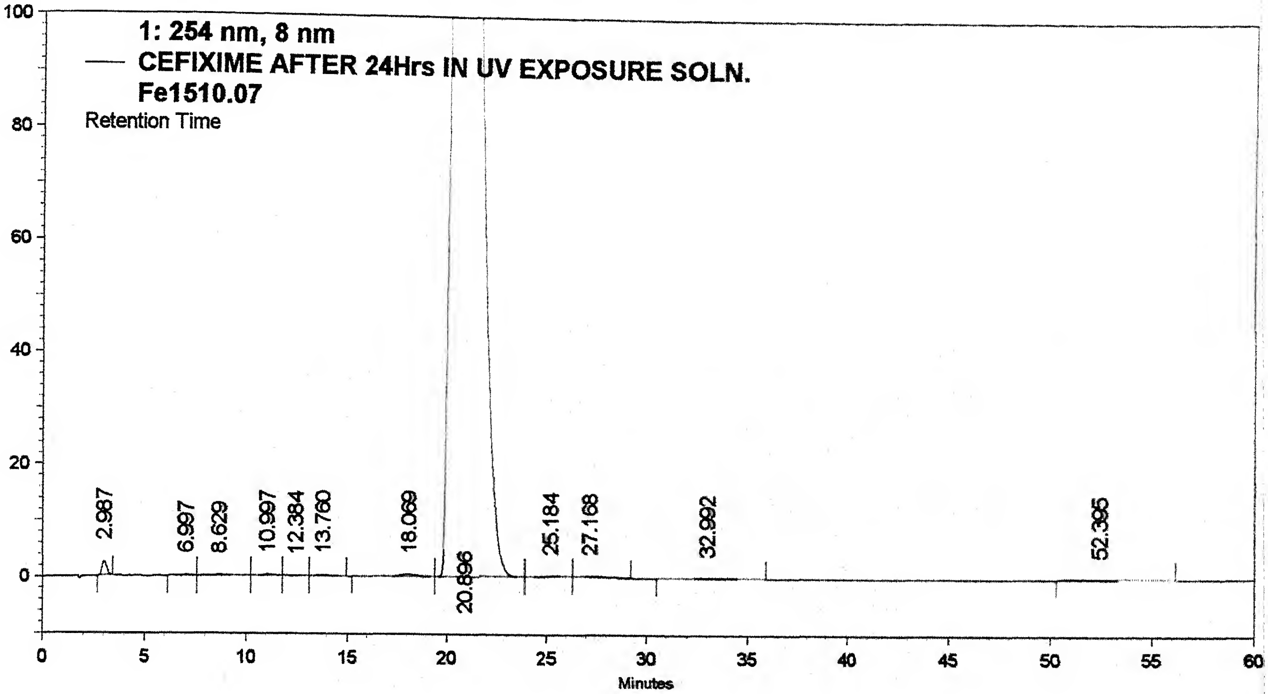


Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 1.000000
Library: C:\CLASS-VP\Spec\CFXuv2.lib

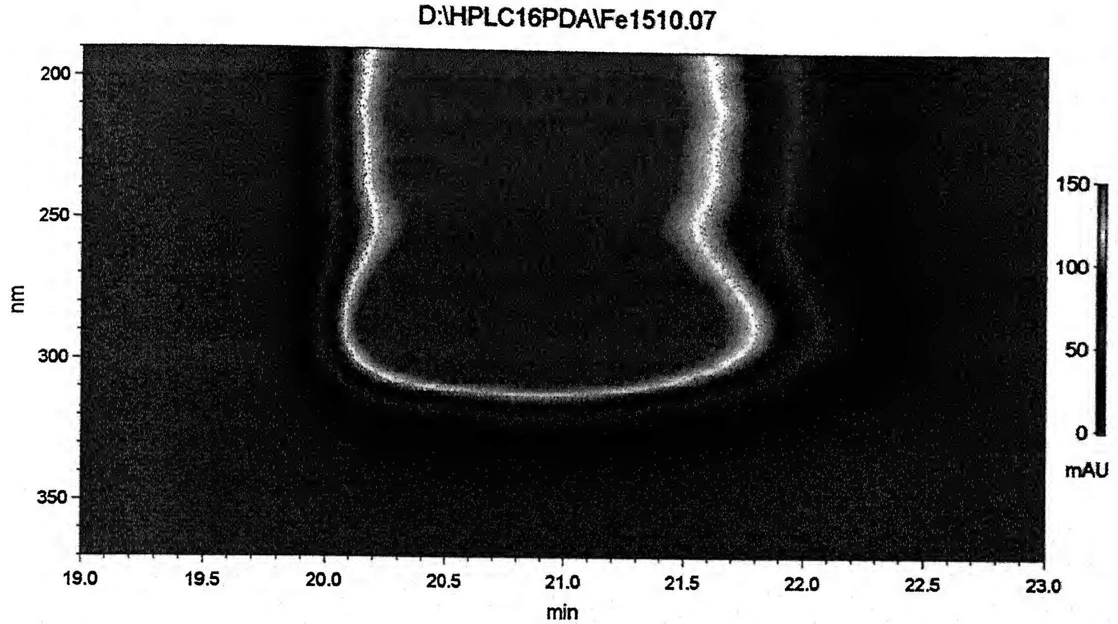
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.07
Acquired Time : 10/15/00 1:49:26 PM
Sample ID : CEFIXIME AFTER 24Hrs IN UV EXPOSURE SOLN.



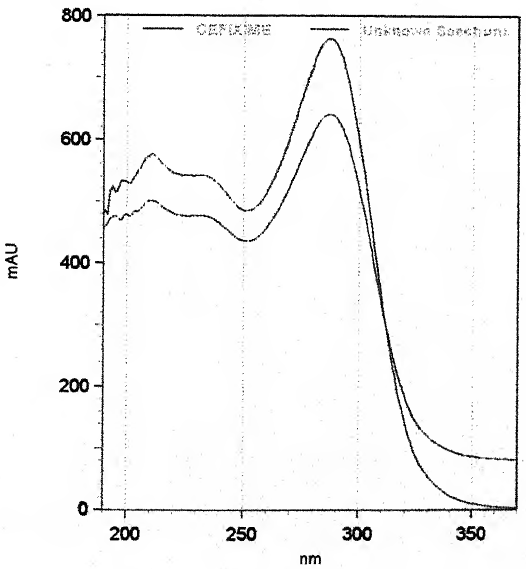
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	2.987	41736	0.22	
2	6.997	8780	0.05	
3	8.629	17662	0.09	
4	10.997	14463	0.08	
5	12.384	5154	0.03	
6	13.760	18798	0.10	
7	18.069	37829	0.20	
8	20.896	18595671	98.86	CEFIXIME
9	25.184	14753	0.08	
10	27.168	18463	0.10	
11	32.992	12975	0.07	
12	52.395	23646	0.13	
Totals		18809930	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.07
Acquired Time : 10/15/00 1:49:26 PM
Sample ID : CEFIXIME AFTER 24Hrs IN UV EXPOSURE SOLN.



Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999928
Library: C:\CLASS-VP\Spec\CFXuv2.lib

Degradation study in Acidic Condition

To check the degradation of Cefixime (B.No. 019/SR/VII/369/39) in Acidic condition. The study carried out in 1N Hydrochloric acid.

Solution preparation :

(a) Stock solution : Weighed 0.2507gm of Cefixime dissolved and diluted in 100ml volumetric flask up to the mark with 1N Hydrochloric acid.

(b) Initial solution :Pipetted 5ml from stock solution immediately after preparation & diluted in 25ml volumetric flask up to the mark with mobile phase .

(c) Subsequent solutions: Pipetted 5ml from stock solution & diluted in 25ml volumetric flask up to the mark with mobile phase after every hour up to 3hrs.

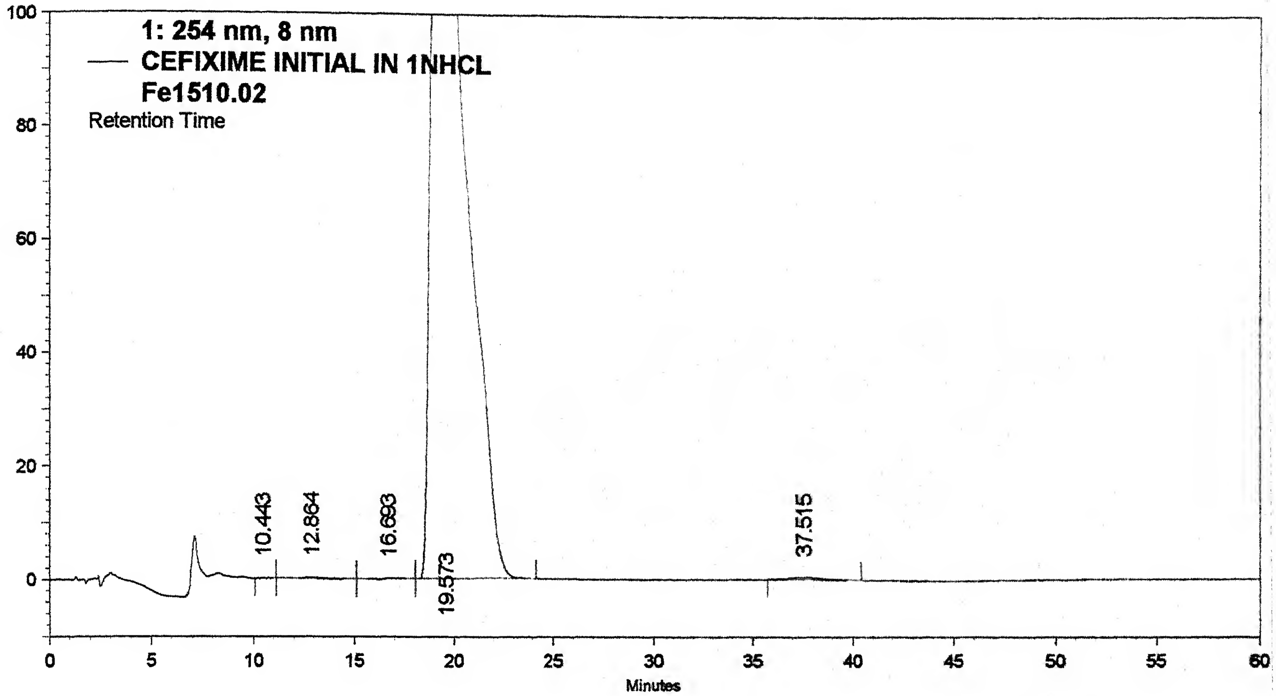
S.No.	Time	Area	% Variance	File Name
01.	Initial	18373475		FE1510.02
02.	After 1 Hr	18257312	0.63	FE1510.03
03.	After 2 Hrs.	18207735	0.90	FE1510.04
04.	After 3 Hrs.	18131286	1.32	FE1510.05

Conclusion :

(1) Spectrum correlation of Cefixime peak at initial and up to 3.0hrs shows correlation is 100%. Which confirms that no other peak is merging with Cefixime.

(2) Contour plot of Cefixime peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefixime.

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.02
Acquired Time : 10/15/00 8:18:03 AM
Sample ID : CEFIXIME INITIAL IN 1NHCL

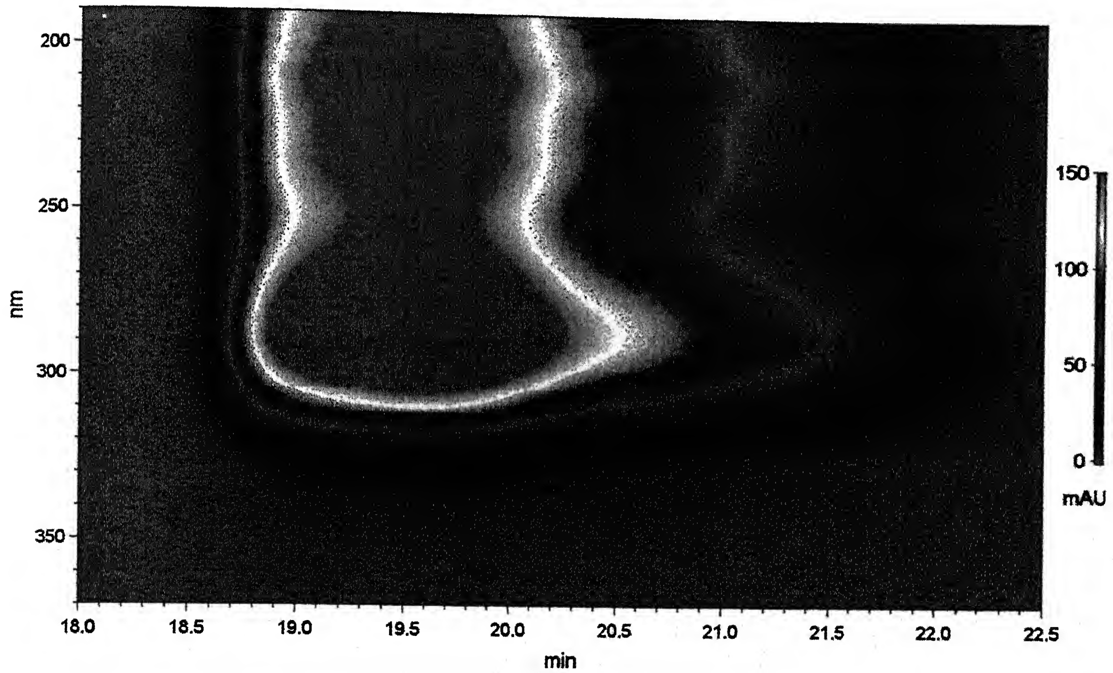


1: 254 nm, 8 nm

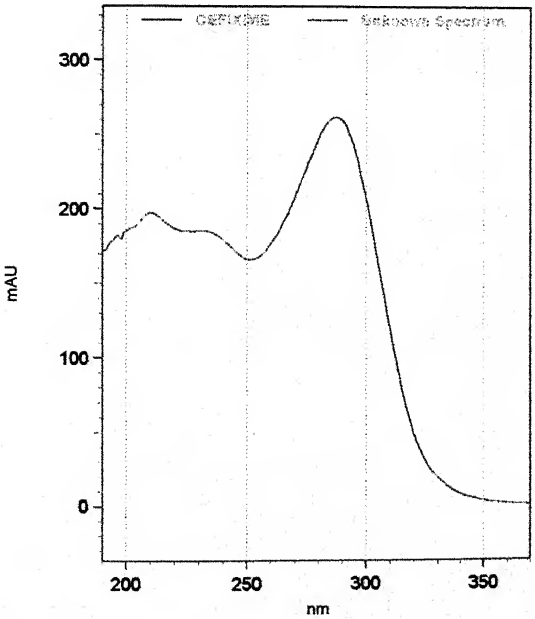
Pk #	Retention Time	Area	Area Percent	Name
1	10.443	4285	0.02	
2	12.864	25548	0.14	
3	16.693	13271	0.07	
4	19.573	18373475	99.45	CEFIXIME
5	37.515	57882	0.31	
Totals		18474461	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.02
Acquired Time : 10/15/00 8:18:03 AM
Sample ID : CEFIXIME INITIAL IN 1NHCL

D:\HPLC16PDA\Fe1510.02

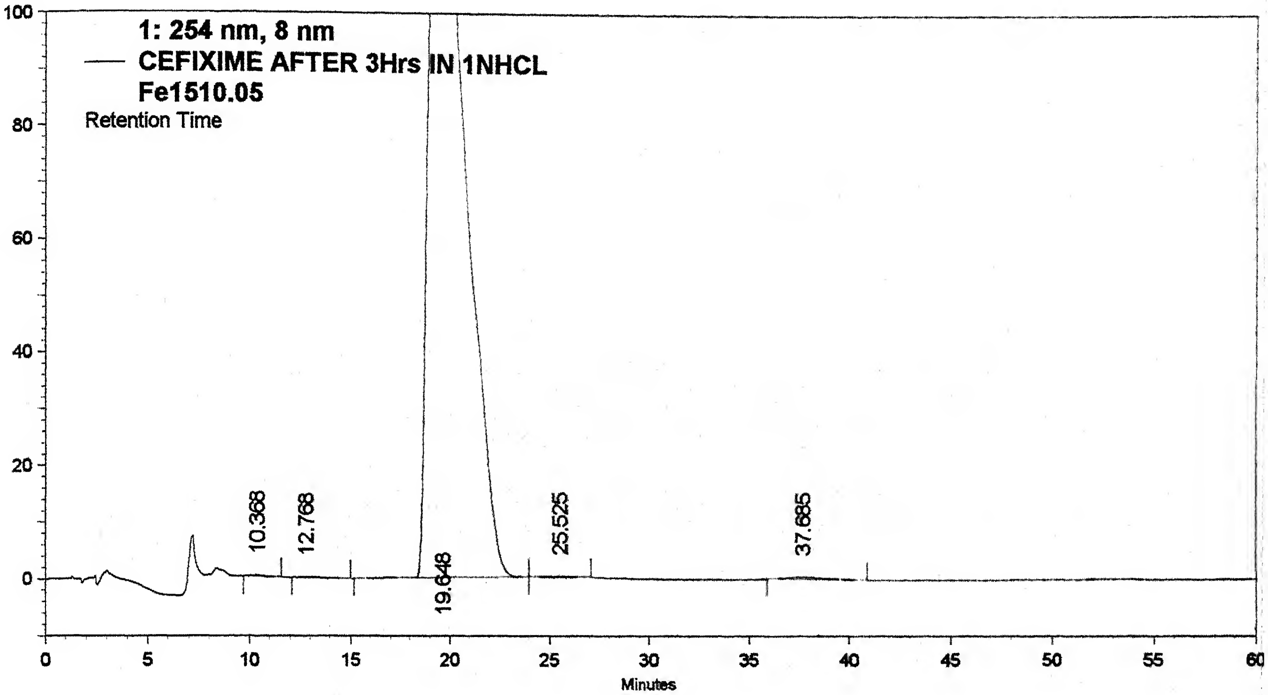


Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 1.000000
Library: C:\CLASS-VP\Spec\CFXHCl.lib

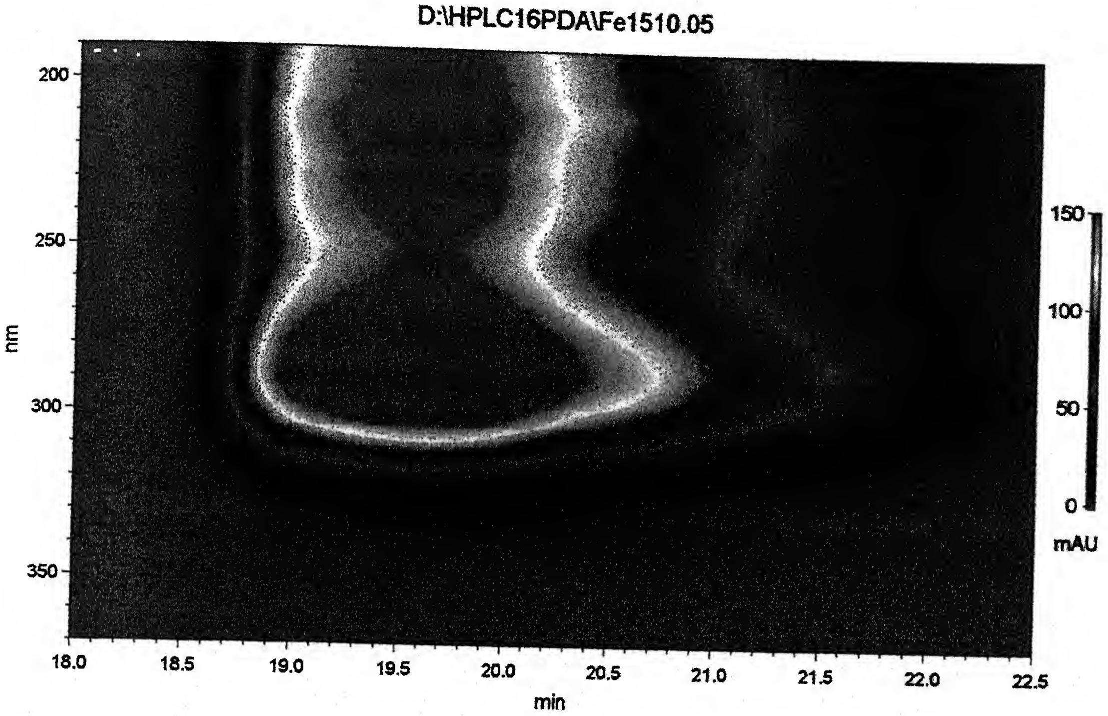
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.05
Acquired Time : 10/15/00 11:38:07 AM
Sample ID : CEFIXIME AFTER 3Hrs IN 1NHCL



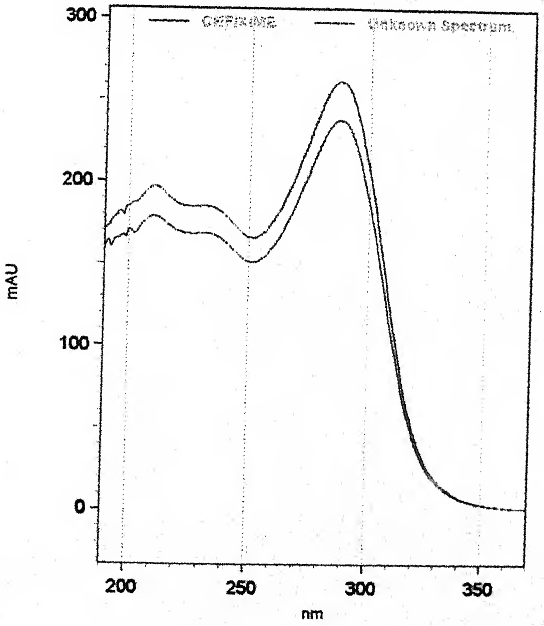
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	10.368	16708	0.09	
2	12.768	19331	0.11	
3	19.648	18138790	99.37	CEFIXIME
4	25.525	17300	0.09	
5	37.685	60912	0.33	
Totals		18253041	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.05
Acquired Time : 10/15/00 11:38:07 AM
Sample ID : CEFIXIME AFTER 3Hrs IN 1NHCL



Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999982
Library: C:\CLASS-VP\Spec\CFXHCl.lib

Degradation study in Alkaline Condition

To check the degradation of Cefixime (B.No. 019/SR/VII/369/39) in Alkaline condition. The study carried out in 1N Sodium Hydroxide.

Solution preparation :

(a) Stock solution : Weighed 0.2518gm of Cefixime in 100ml Volumetric flask. Added 5ml of 1N NaOH & make up to the mark with mobile phase and sonicate for dissolution.

(b) Initial solution :Pipetted 5ml from stock solution immediately after preparation & diluted in 25ml volumetric flask up to the mark with mobile phase.

(c) Subsequent solutions: Pipetted 5ml from stock solution & diluted in 25ml volumetric flask up to the mark with mobile phase. after every hour up to 3hrs.

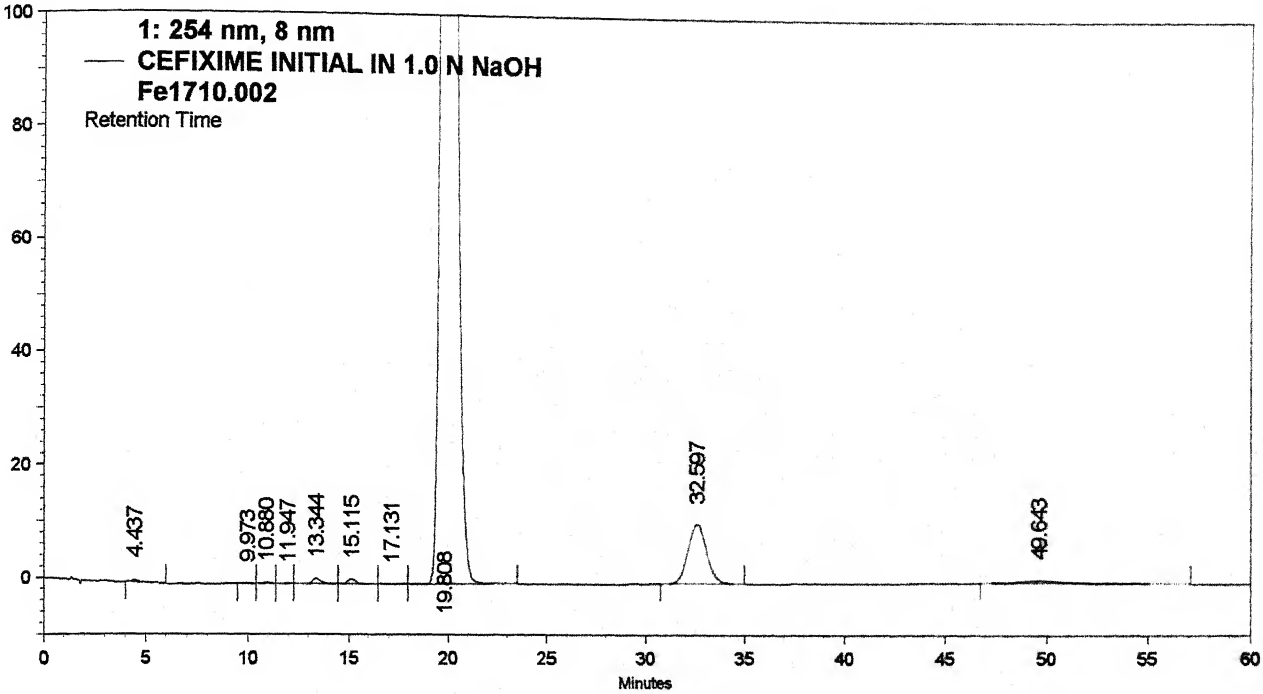
S.No.	Time	Area	% Variance	File Name
1.	Initial	16771177	-	FE1710.002
2.	After 1 hr	8258920	50.75	FE1710.003
3.	After 2 hr	5031577	70.0	FE1710.004
4.	After 3 hr	2760714	83.53	FE1610.005

Conclusion :

(1) Spectrum correlation of Cefixime peak at initial & after 3.0 hrs shows correlation is 100%. Which confirms that no other peak is merging with Cefixime.

(2) Contour plot of Cefixime peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefixime.

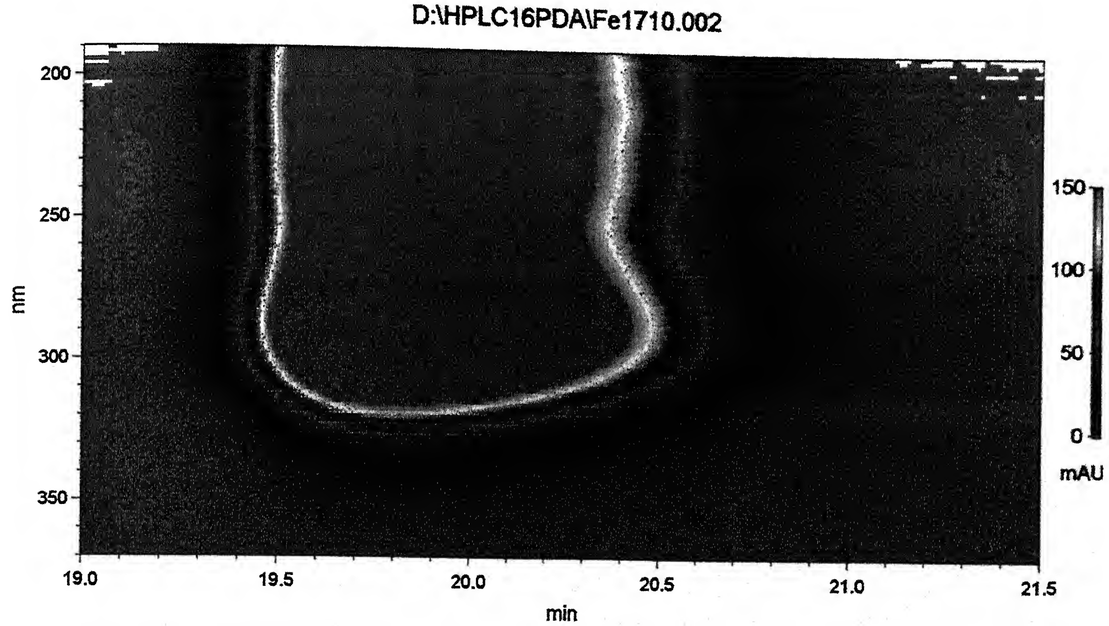
Method Name : **C:\CLASS-VP\Methods\CFXPDA.met**
File Name : **D:\HPLC16PDA\Fe1710.002**
Acquired Time : **10/17/00 12:39:54 PM**
Sample ID : **CEFIXIME INITIAL IN 1.0 N NaOH**



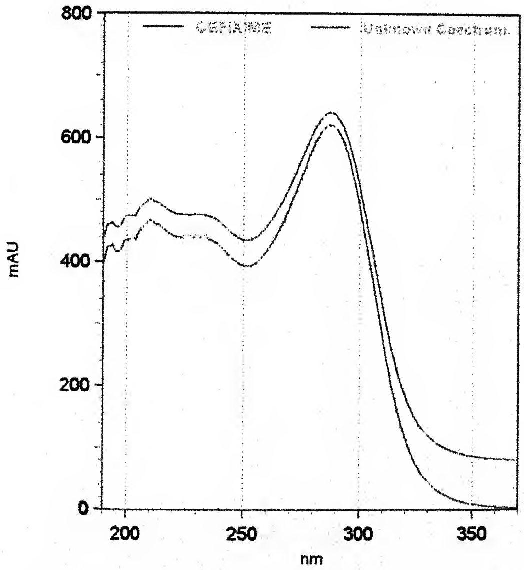
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	4.437	13016	0.07	
2	9.973	2851	0.02	
3	10.880	7465	0.04	
4	11.947	1739	0.01	
5	13.344	32112	0.18	
6	15.115	26448	0.15	
7	17.131	2959	0.02	
8	19.808	16771177	94.90	CEFIXIME
9	32.597	704638	3.99	
10	49.643	110365	0.62	
Totals		17672770	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1710.002
Acquired Time : 10/17/00 12:39:54 PM
Sample ID : CEFIXIME INITIAL IN 1.0 N NaOH

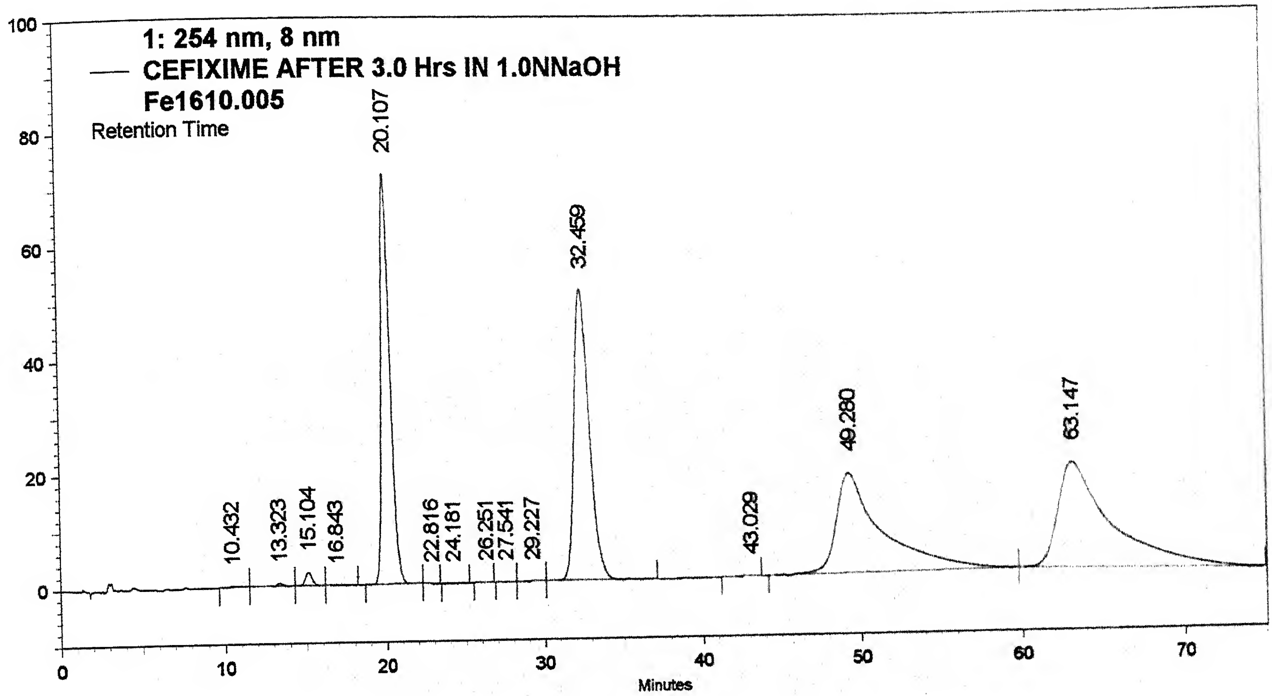


Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999999
Library: C:\CLASS-VP\Spec\CFXbasic.lib

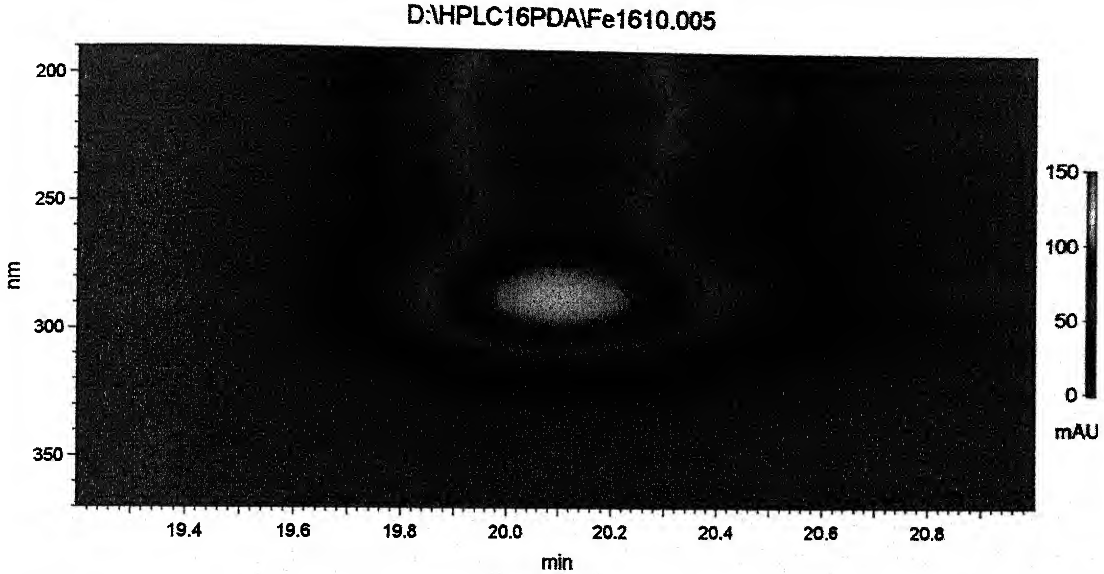
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1610.005
Acquired Time : 10/17/00 4:07:10 PM
Sample ID : CEFIXIME AFTER 3.0 Hrs IN 1.0NNaOH



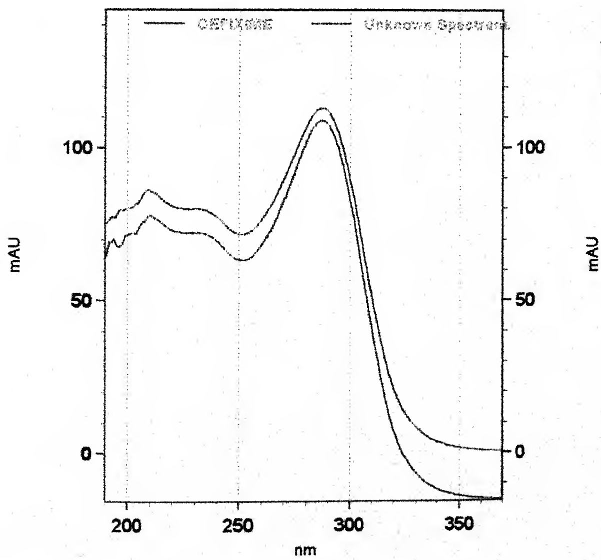
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	10.432	8768	0.06	
2	13.323	18804	0.13	
3	15.104	71253	0.50	
4	16.843	4830	0.03	
5	20.107	2760714	19.43	CEFIXIME
6	22.816	2058	0.01	
7	24.181	3932	0.03	
8	26.251	2742	0.02	
9	27.541	2664	0.02	
10	29.227	3894	0.03	
11	32.459	3431916	24.16	
12	43.029	7332	0.05	
13	49.280	3578050	25.18	
14	63.147	4310362	30.34	
Totals		14207319	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1610.005
Acquired Time : 10/17/00 4:07:10 PM
Sample ID : CEFIXIME AFTER 3.0 Hrs IN 1.0NNaOH



Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999965
Library: C:\CLASS-VP\Spec\CFXbasic.lib

Degradation study in Oxidative Condition

To check the degradation of Cefixime in Oxidative condition. The study carried out in Hydrogen peroxide (8.2%).

Solution preparation :

(a) Stock solution: Weighed 0.2509gm of Cefixime in 100ml volumetric flask. Add 5ml Hydrogen peroxide (8.2%) then make up to the mark with mobile phase .

(b) Initial solution :Pipetted 5ml from stock solution immediately after preparation & diluted in 25 ml volumetric flask up to the mark with mobile phase.

(c) Subsequent solutions: Pipetted 5ml from stock solution & diluted in 25ml volumetric flask make up to the mark with mobile phase after every hrs up to 3.0 hrs.

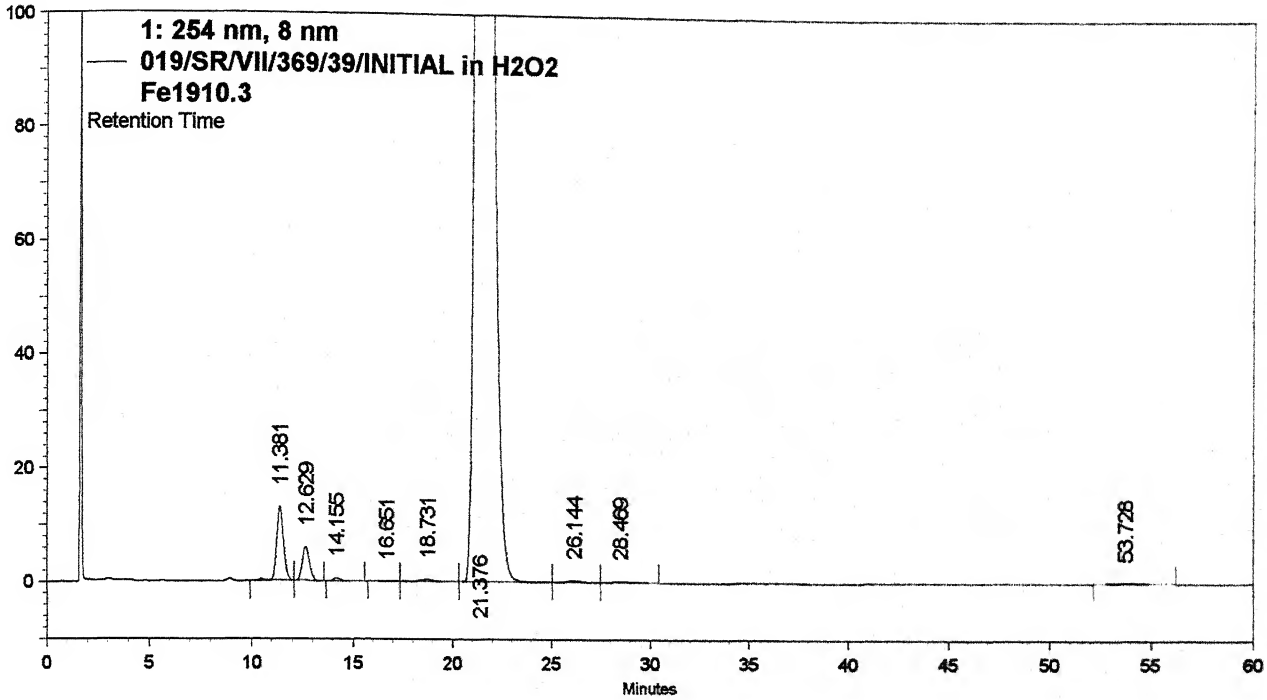
S.No.	Time	Area	% Variance	File Name
1.	Initial	17756751		FE1910.3
2.	After 1hr	17261861	2.79	FE1910.4
3.	After 2 hr	16749709	5.67	FE1910.5
4.	After 3 hr	16220554	8.65	FE1910.6

Conclusion :

(1) Spectrum correlation of Cefixime peak at initial & after 3hrs shows correlation is 100%. Which confirms that no other peak is merging with Cefixime.

(2) Contour plot of Cefixime peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefixime.

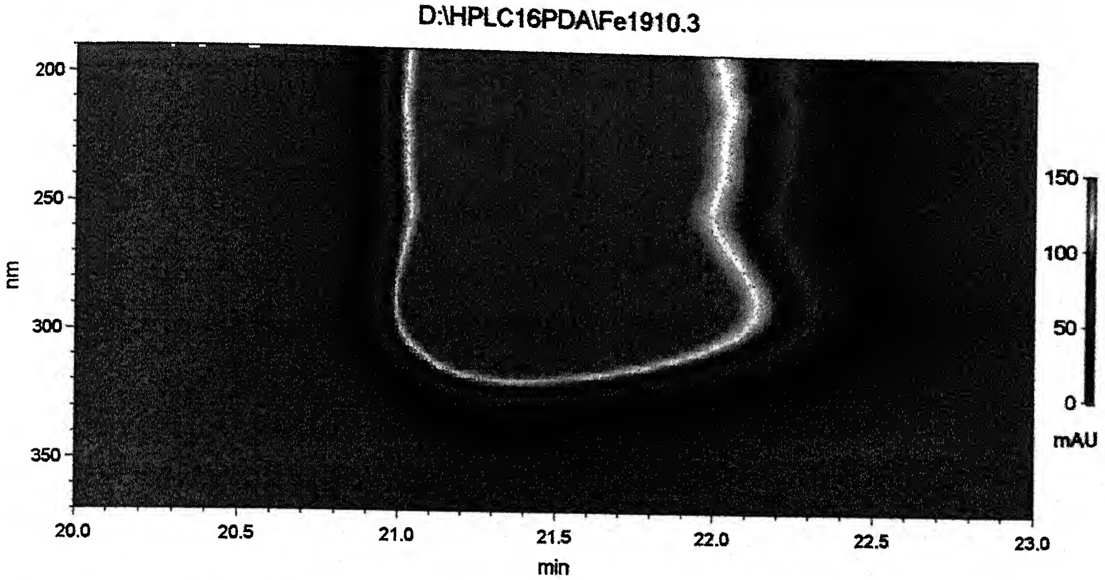
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1910.3
Acquired Time : 10/19/00 12:29:54 PM
Sample ID : 019/SR/VII/369/39/INITIAL in H2O2



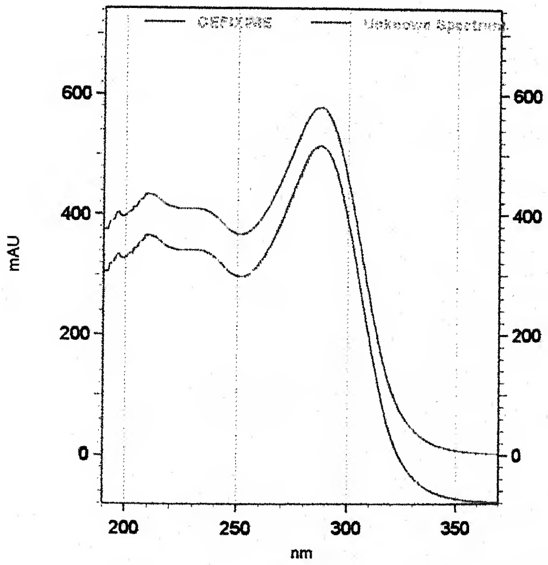
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	11.381	316410	1.73	
2	12.629	150887	0.82	
3	14.155	20512	0.11	
4	16.651	6336	0.03	
5	18.731	24456	0.13	
6	21.376	17756751	96.87	CEFIXIME
7	26.144	18820	0.10	
8	28.469	13578	0.07	
9	53.728	22363	0.12	
Totals		18330113	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1910.3
Acquired Time : 10/19/00 12:29:54 PM
Sample ID : 019/SR/VII/369/39/INITIAL in H2O2

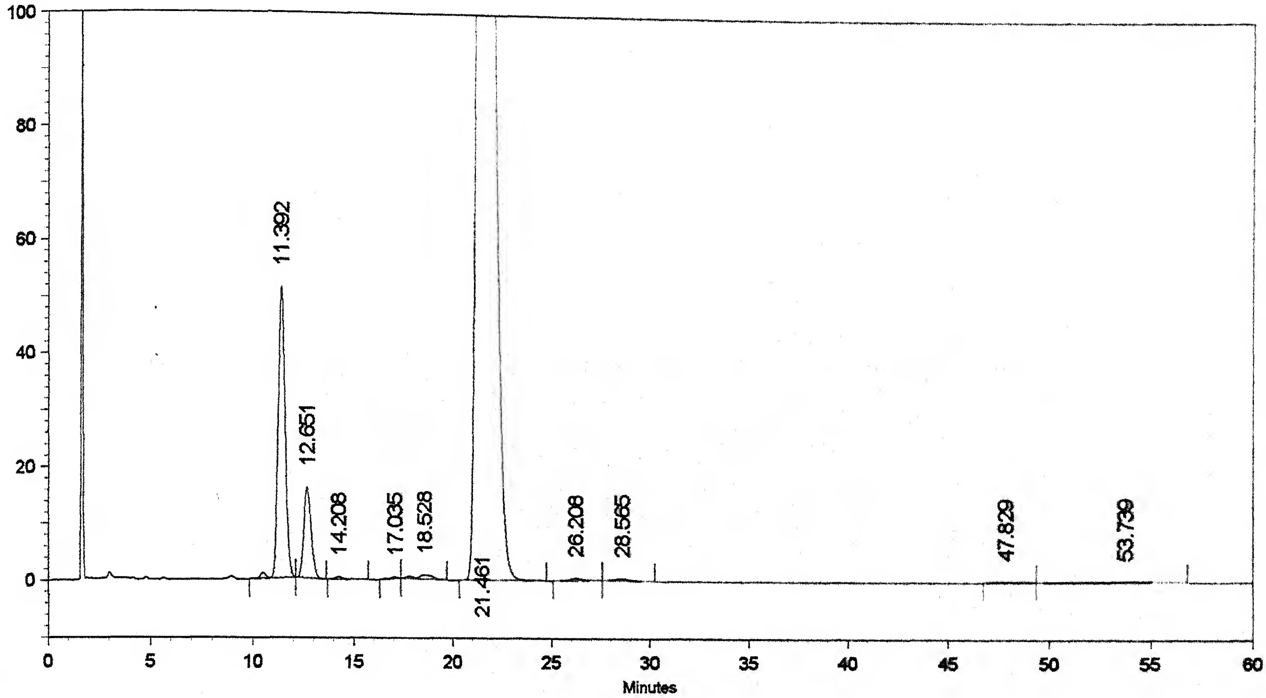


Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 1.000000
Library: C:\CLASS-VP\Spec\CFXox.lib

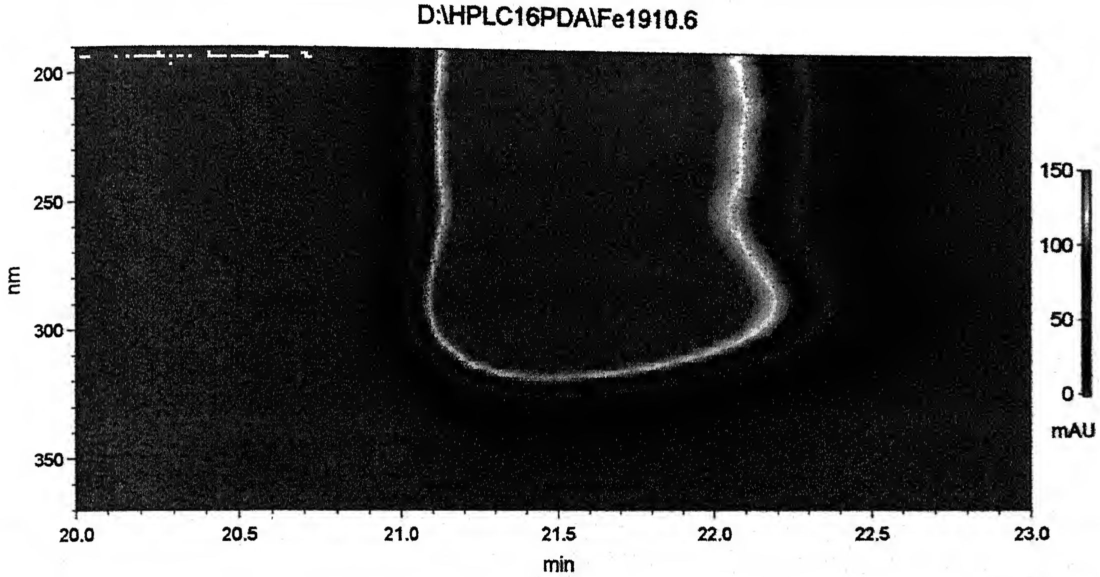
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1910.6
Acquired Time : 10/19/00 3:41:13 PM
Sample ID : After 3.0 hrs in H2O2



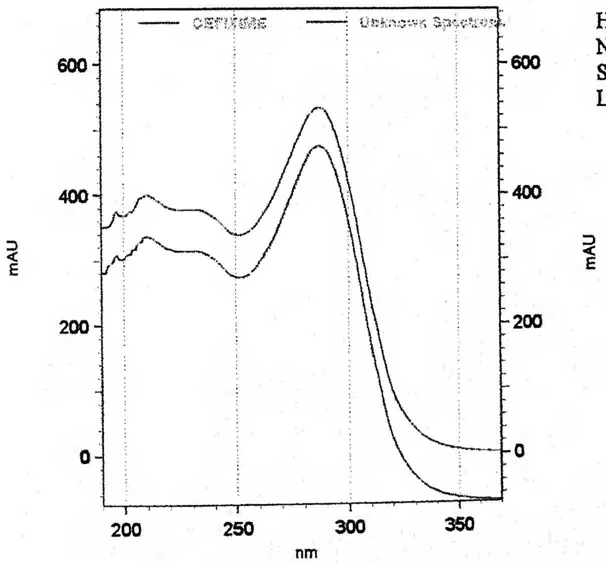
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	11.392	1226071	6.80	
2	12.651	405956	2.25	
3	14.208	19710	0.11	
4	17.035	9496	0.05	
5	18.528	51889	0.29	
6	21.461	16220554	89.95	CEFIXIME
7	26.208	26641	0.15	
8	28.565	23431	0.13	
9	47.829	4552	0.03	
10	53.739	45052	0.25	
Totals		18033352	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1910.6
Acquired Time : 10/19/00 3:41:13 PM
Sample ID : After 3.0 hrs in H2O2



Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999994
Library: C:\CLASS-VP\Spec\CFXox.lib

Degradation study on Thermal Condition

To check the degradation of Cefixime after heating at 50°C ± 1°C for 3 hours.

Solution preparation :

(a) Stock solution :Weighed 0.2501gm of Cefixime (B.No. 019/SR/VII/369/39) dissolved and diluted in 100ml volumetric flask up to the mark with mobile phase. Keep the stock solution in water bath at 50°C for 3.0 hrs.

(b) Initial solution :Pipetted 5ml from stock solution before keeping In to water in 25ml volumetric flask up to the mark with mobile phase.

(c) Subsequent solutions: Pipetted 5ml from stock solution & diluted in 25ml volumetric flask up to the mark with mobile phase after every hour up to 3.0 hrs.

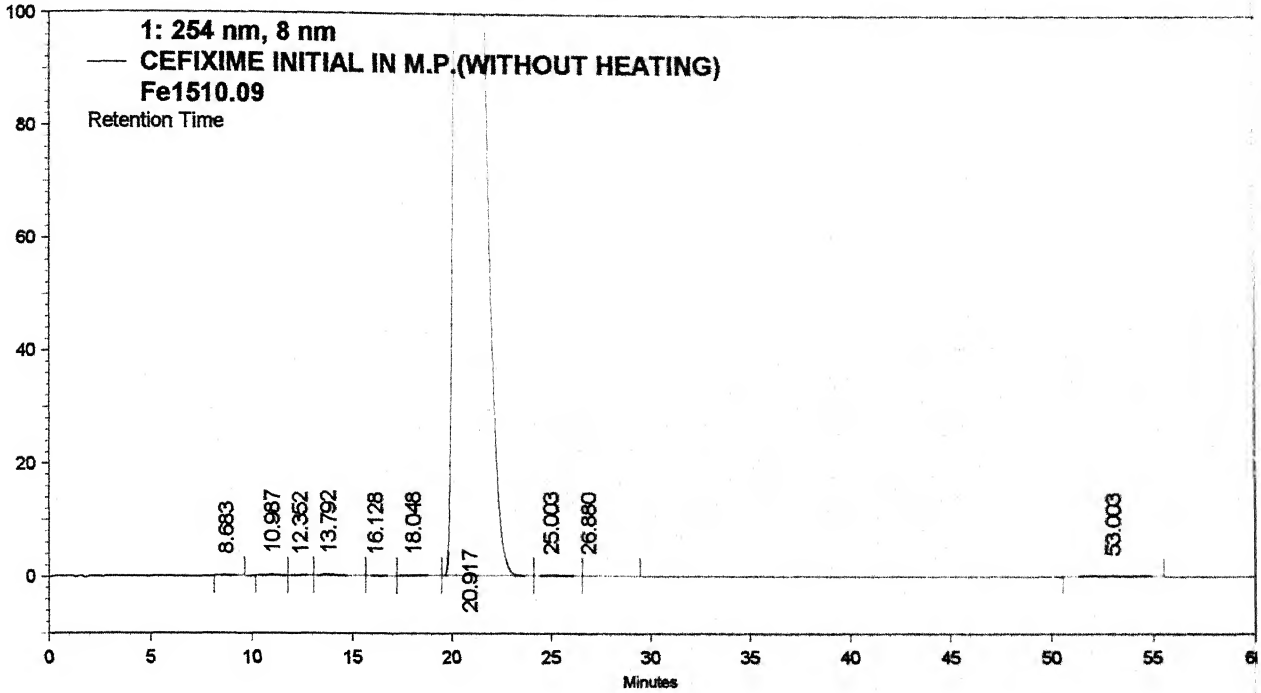
S.No.	Time	Area	% Variance	File Name
1.	Initial	18381588		FE1510.09
2.	After 1Hr.	17867728	2.8	FE1510.10
3.	After2 Hrs.	17656600	3.94	FE1510.11
4.	After3 Hrs.	17456336	5.03	FE1510.12

Conclusion :

(1) Spectrum correlation of Cefixime peak at initial & after 3.0 hrs shows correlation is 100%. Which confirms that no other peak is merging with Cefixime.

(2) Contour plot of Cefixime peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefixime.

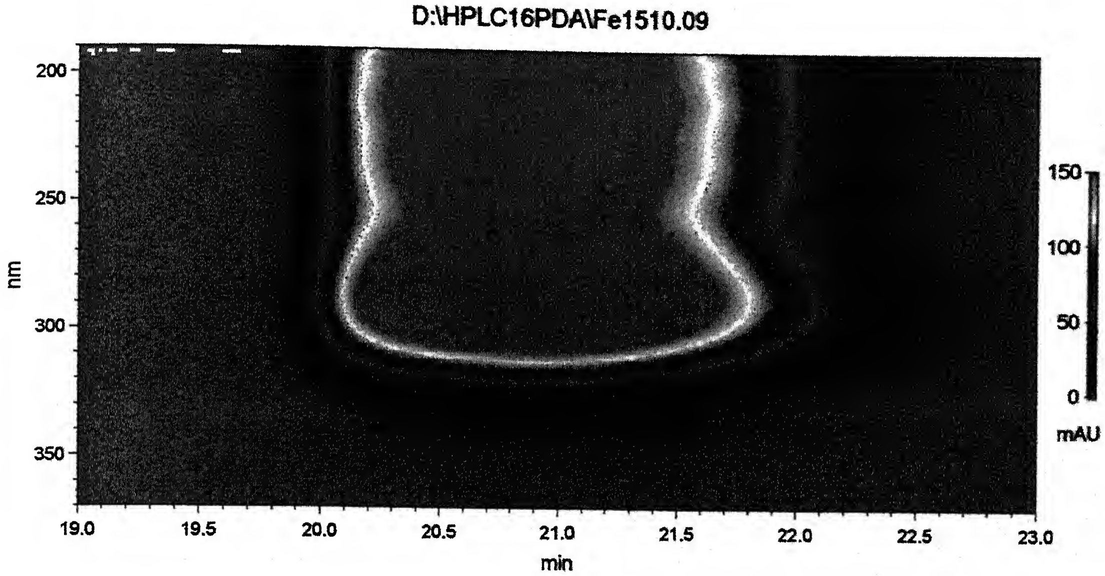
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.09
Acquired Time : 10/15/00 3:56:23 PM
Sample ID : CEFIXIME INITIAL IN M.P.(WITHOUT HEATING)



1: 254 nm, 8 nm

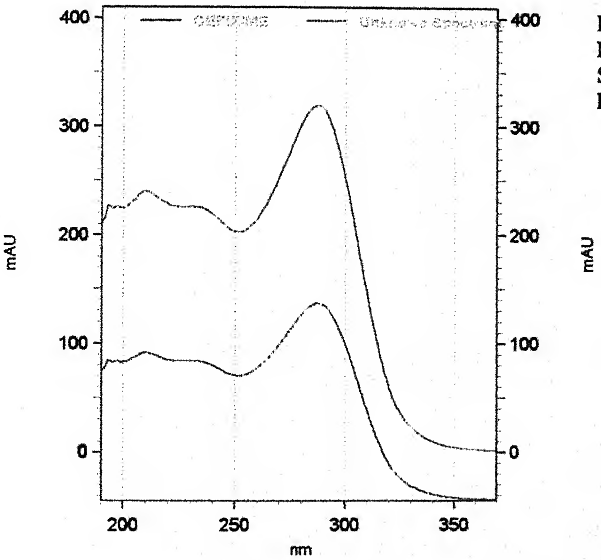
Pk #	Retention Time	Area	Area Percent	Name
1	8.683	7678	0.04	
2	10.987	12298	0.07	
3	12.352	3098	0.02	
4	13.792	19802	0.11	
5	16.128	3190	0.02	
6	18.048	12601	0.07	
7	20.917	18381588	99.51	CEFIXIME
8	25.003	8673	0.05	
9	26.880	4240	0.02	
10	53.003	18277	0.10	
Totals		18471445	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.09
Acquired Time : 10/15/00 3:56:23 PM
Sample ID : CEFIXIME INITIAL IN M.P.(WITHOUT HEATING)



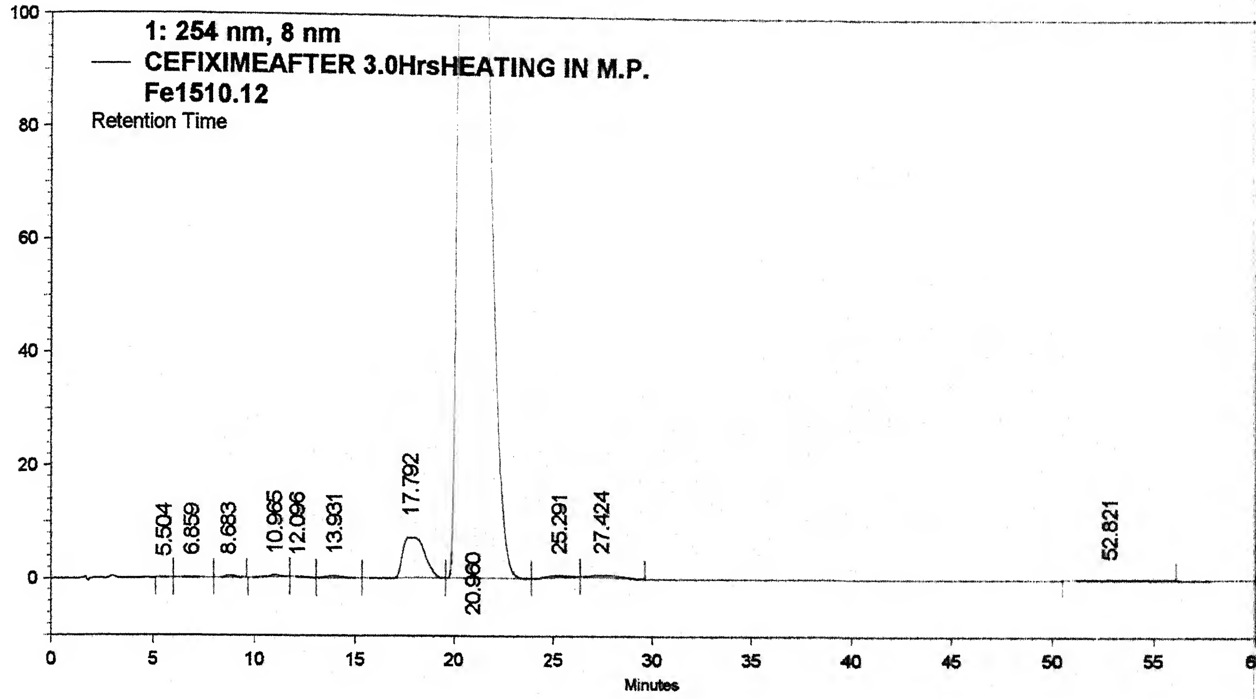
Peak Name: CEFIXIME

Number of Hits: 1



Hit 1
Name: CEFIXIME
Similarity Index: 1.000000
Library: C:\CLASS-VP\Spec\CFXHeat.lib

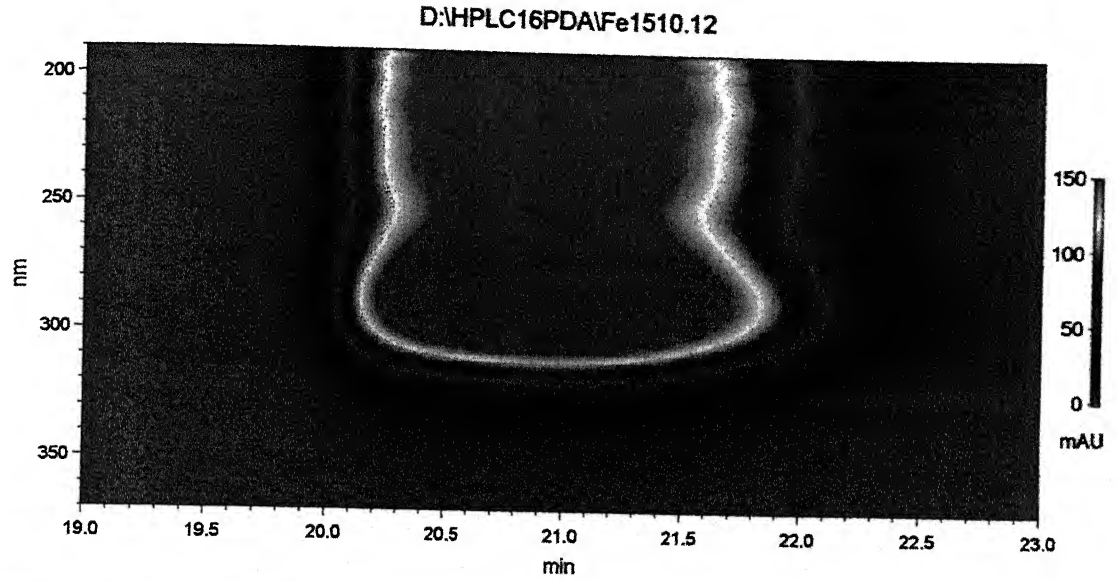
Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.12
Acquired Time : 10/15/00 7:18:12 PM
Sample ID : CEFIXIMEAFTER 3.0HrsHEATING IN M.P.



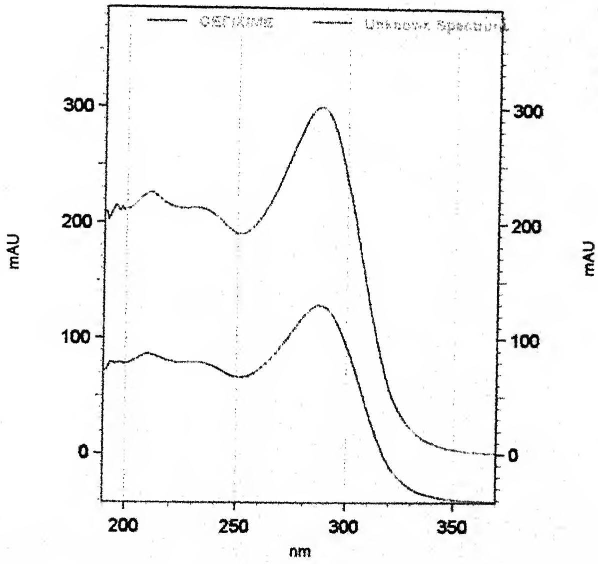
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	5.504	2948	0.02	
2	6.859	7079	0.04	
3	8.683	17540	0.10	
4	10.965	21426	0.12	
5	12.096	6214	0.03	
6	13.931	27359	0.15	
7	17.792	556296	3.06	
8	20.960	17456336	95.92	CEFIXIME
9	25.291	33871	0.19	
10	27.424	50486	0.28	
11	52.821	19624	0.11	
Totals		18199179	100.00	

Method Name : C:\CLASS-VP\Methods\CFXPDA.met
File Name : D:\HPLC16PDA\Fe1510.12
Acquired Time : 10/15/00 7:18:12 PM
Sample ID : CEFIXIMEAFTER 3.0HrsHEATING IN M.P.



Peak Name: CEFIXIME **Number of Hits: 1**



Hit 1
Name: CEFIXIME
Similarity Index: 0.999977
Library: C:\CLASS-VP\Spec\CFXHeat.lib

IMPURITY PROFILING OF CEFIXIME

Impurity profile (i.e. related substance test) is the most important test for any compound. Because, now a day it is mandatory that any impurity, which is in amount 0.1% or more should be isolated and characterize. Impurity profile test is carried out by liquid chromatography method. This test provide valuable information about quality of product and its impurity during stability study. Official Monograph of Cefixime is available in U.S. and European Pharmacopoeia, Out of these two Pharmacopoeias only European Pharmacopoeia mentioned the test for related substance.

As per European Pharmacopoeia, the method for related substances and assay is same (i.e. By HPLC), but for related substance continue the chromatography for three times the retention time of the Cefixime peak. We have tried European as well as our gradient HPLC method for checking related substance in Cefixime. We found that our method gives not only better resolution between impurities and Cefixime, but one additional impurity also detected. Therefore, for impurity profile purpose we have adopted our own method.

EXPERIMENTAL SECTION

Materials

Cefixime B.No. CXM/080005 of Lupin ltd. was used and all other chemicals used were of reagent grade. Water was purified by milli-Q System.

Procedures

The following instruments were used for the impurity profiling of cefixime.

Instruments : IR spectra were recorded on Perkin-Elmer 1650(USA) and Shimadzu FT-IR 8201(Japan) model, NMR spectra were recorded on Bruker DRX-200 MHz(Switzerland) and Mass spectra were recorded on PE –SCIEX API-3000(USA) triple quadruple. The pH of solution was measured using Control Dynamic (India). The balance was Mettler –AE-250 (Switzerland).

Analytical System: Shimadzu binary gradient HPLC equipped with

- System controller – SCL 10 ATvp
- Two pumps - LC – 10ATvp
- Degasser unit - DGU – 14A
- UV/VIS. Detector – SPD – 10 Avp
- Autosampler - SIL – 10 ADvp fitted with 50 μl sample loop
- Column oven - 10 Asvp
- Data processor - Class-VP –5.03 software
- Column - Inertsil ODS – 3V (250mm * 4.6mm, 5.0μ Spherical particle)

Mobile Phase - 0.05m Ammonium Acetate (Buffer): Methanol

Solution – A - Buffer: Methanol (95: 05)

Solution – B - Buffer: Methanol (50: 50)

Wavelength - 254 nm

Flow rate - 1.5 ml/min

Analytical Gradient Condition:

S.N.	Time	Event	Value	Flow ml/min
1	0.01	B.conc	0.0	1.5
2	5.00	B.conc	5.0	1.5
3	10.00	B.conc	15.0	1.5
4	20.00	B.conc	30.0	1.5
5	30.00	B.conc	50.0	1.5
6	40.00	B.conc	75.0	1.5
7	50.00	B.conc	100.0	1.5
8	60.00	B.conc	100.0	1.5
9	65.00	B.conc	0.0	1.5
10	68.00	B.conc	0.0	1.5

Prparative HPLC :

- Two pumps - LC- 8A
- One UV/Vis detector - SPD 10-A
- Column -WatersX-terra C-18 (300mm * 19mm,7.0μ)
  -G.L.Science-Inertsil-
  C-18(250mm*20mm,8.0μ)
  -Waters Spherisorb C-18 (250mm
  *20mm,10μ)

Medium Presser Liquid Chromatography:

- System controller Labomat-VS-200
- Pump - Laboprep MD-50
- UV detector - UVIS-201

- Column - 700mm * 50mm Glass (Packed by C-18, 20-40 μm particle.)

- **Lyophilizer :** Virtis EL2500.

EXPERIMENTAL WORK

Isolation of Cefixime impurities-A,B,and D at RRT-0.744, 1.455,AND1.33 (RRT0.74,0.79,0.91,0.93(A),1.27(D),1.39,1.44(B)

Weighed 5.0gms.Cefixime sample B.No. CXM/080005 in 250.0ml beaker, add 100.0ml water and heated at 80°c for 5hrs. to enrich the impurity. After checking LC-traces of water layer and solid, it was found that all desired impurity present in water layer, therefore, water layer was lyophilized.

Prep.HPLC system using following condition employed for further purification of lyophilized mass.

Column	- Inertsil ODS-3, C-18 (250mm * 20mm,8μm particle)
Mobile phase	- 0.05 m Ammonium Acetate: Methanol
	Solution A - Buffer: Methanol (95: 05)
	Solution B - Buffer: Methanol (50: 50)
pH	- 4.2 (Adjusted with H_3PO_4)

Flow - 15.0ml/min

Wavelength - 254nm

Column oven Temp. - 40°C

All the fractions collected were monitored using analytical system and the purity was found in the range of 75-79 %. The fractions containing impurity were concentrated on rotavapour at bath temperature 30-32°c using high vacuum to distill the methanol. The aqueous solution was lyophilized, thereafter purified up to 98 % by reloading it on prep. HPLC using same parameters mentioned as above. These repurified fractions were concentrated on rotavapour and lyophilized thrice to remove ammonium acetate. The solid mass was taken for purity evaluation and other spectroscopic studies.

Conclusion – Impurity – A(0.74,0.79,0.91,and 0.93RRT)

The structure of impurities A, A1, A2, and A3 were found as isomeric impurity of cefixime formed due to cyclization taken place between 2-caroxyl and 3-vinyl group and opening of beta lactam ring. The distereoisomerism was explained on the basis of isomerization of the γ-lactone methyl.

PMR Spectra

The PMR spectra of impurity A in MeOD confirm the structure of above-mentioned impurity.

S.No.	Chemical shift (ppm)	Multiplicity	Proton assignment
1.	9.4	d	CONH
2.	7.3	b	NH_2
3.	7.22	s	H of Ar
4.	5.98	b	-NH-
5.	5.52	d	H of Lactone
6.	4.95	s	7-H
7	4.80	s	6-H
8.	4.6	s	-O-CH2
9.	3.60 – 3.80	q	$-S-CH_2$
10.	1.45	d	$-CH_3$

At δ 1.45 and 5.52 new proton signals are observed, it is because of –CH3 and –CH, and the C-7(5) proton signals shifted upfield from 5.85 to 4.95ppm. There are no proton signals for Vinyl group. Hence, impurity is assigned as lactam ring opened lactone formed due to lactonization between 2-carboxyl and 3-vinyl group.

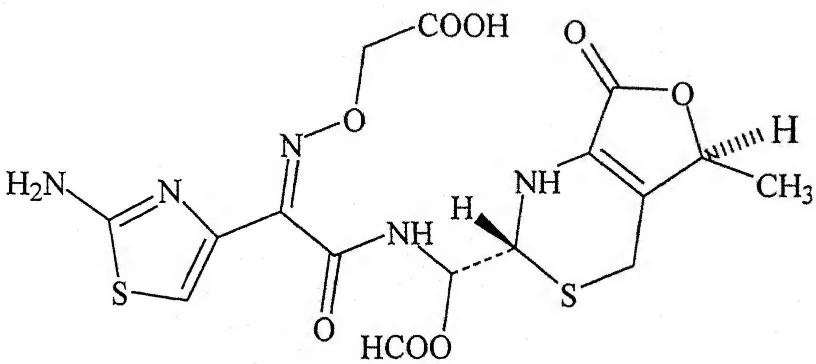
Mass Spectra

The assignment to the molecular ion peaks is given below:

S.N.	m/z amu	Structural
1	472 (M+H)+	Molecular ion

Summary

The unidentified impurities of A, A1, A2, andA3 ware isolated from Cefixime using preparative HPLC. The structures of these impurities were assigned on the basis of PMR and Mass Spectra. They were characterized as isomeric impurity of 2-((2R)-5-Methyl-7-oxo(5-hydro-1H,2H,4H-furano[3,4-d]1,3-thiazin-2-yl))(2S)-2-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy)prop-2-enoylamino] acetic acid.



Conclusion –Impurity-B (1.39 and 1.33RRT)

The structure of impurities B, B1, B2, and B3 were assigned as distereoisomers formed due to lactonization between 2-carboxyl and 3-vinyl group, β lactam ring opening, and the elimination of carboxylic group.

PMR Spectra

The PMR spectra of impurity B in MeOD confirm the structure of above-mentioned impurity.

S.No.	Chemical shift (ppm)	Multiplicity	Proton assignment
1.	9.4	d	-CONH-
2.	7.3	b	$-NH_2$
3.	7.22	s	H of Ar
4.	5.98	b	7-H
5.	5.52	d	H of Lactone
6.	4.81	q	6-H
7.	4.6	s	$-O-CH_2$
8.	3.9	d	$7-CH_2$
9.	3.50 – 3.78	q	$-S-CH_2$
10.	1.51	d	$-CH_3$

The NMR signals of $–CH_3$ and –CH are also observed, and $[M+H]^+$ at 428 indicate that this impurity is formed due to decarboxylation of impurity **A**.

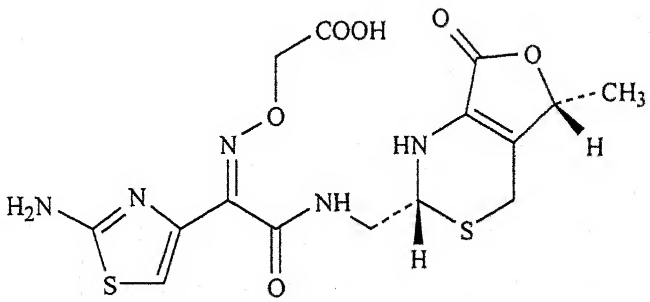
Mass Spectra

The assignment to the molecular ion peaks are given below:

S.N.	m/z amu	Structural
1	428 (M+H)+	Molecular ion

Summary

The unidentified impurities B, B1, B2, B3 were isolated from Cefixime using MPLC and preparative HPLC. The structures of these impurities were assigned on the basis of PMR and Mass Spectra. They were characterized as isomer of 2-(2-{N-[((2R,5R)-5-Methyl-7-oxo(5-hydro-1H,2H,4H-furano[3,4-d]1,3-thiazin-2-yl))methyl]carbamoyl}(1Z)-2-(2-amino(1,3-thiazol-4-yl))-1-azavinyloxy)acetic acid



Conclusion -impurity-D(1.27 RRT)

The structure of impurity D was assigned as an E-isomer of cefixime.

PMR Spectra

The PMR spectra of impurity D in MeOD indicated to an E-isomer of cefixime as given below:

S.No.	Chemical shift (ppm)	Multiplicity	Proton assignment
1.	9.5	d	-CONH
2.	7.60	s	H of Ar
3.	7.28	b	NH_2
4.	6.96-7.1	m	Vinyl
5.	5.80-5.85	dd	7-H
6.	5.60	d	Vinyl
7.	5.38	d	Vinyl
8.	5.20	d	6-H
9.	4.65	s	$-O-CH_2$
10.	3.53 - 3.9	q	$-S-CH_2$

The proton signal of –SCH is shifted upfield from 6.72 to 7.60 indicate E-isomer of ceixime.

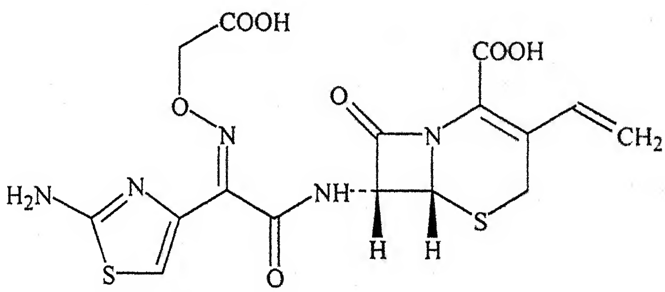
Mass Spectra

The assignment to the molecular ion peak is given below:

S.N.	m/z amu	Structural
1	476 (M+Na)+	Molecular ion
2	454 (M+H)+	Molecular ion

Summary

The unidentified impurity D was isolated from Cefixime using MPLC and preparative HPLC. The structure of this impurity was assigned on the basis of PMR and Mass Spectra. It was identified as E-isomer of cefixime. 6-[(2Z)-2-(2-amino(1,3-thiazol-5-yl))-3-aza-3-(carboxymethoxy)prop-2-enoylamino]-5-oxo-3-vinyl-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid.



Isolation of cefixime impurity-C(1.21RRT)

Weighed 1.5 gm. of Cefixime sample B.No.CXM/080005 in 100.0ml beaker, add 20.0ml of water and pH was adjusted to 11.5 with 8.0M NaOH. This was heated at 35°C for 15min. and then neutralized it with 5.0M HCl. This solution was filtered and loaded to prep.HPLC using following gradient condition.

Column - Inertsil ODS-3 (250mm * 20mm,8μm particle size)

Buffer - Ware + Acetic acid (0.4%)

Solution A - Buffer: Acetonitrile (70: 30)

Solution B - Buffer: Acetonitrile (50: 50)

pH - As such

Wavelength - 254nm

Flow - 15.0ml/min.

S.N.	Time	Event	Value	Flow ml/min
1	0.01	B.conc	0.0	15.0
2	10.00	B.conc	0.0	15.0
3	50.00	B.conc	100.0	15.0
4	55.00	B.conc	00.0	15.0
5	60.00	B.conc	00.0	15.0
6	61.00	B.conc	Stop	00

All the fractions collected were monitored by analytical system and the purity was found in the range of 93-95%. The fractions containing impurity were concentrated on rotavepour at bath temperature 30-32°C using high vacuum to distill the Acetonitrile. The solution was lyophilized twice to remove Acetic acid. The solid mass was taken for purity evaluation and other spectroscopic studies.

Conclusion (1.21RRT)

The structure of impurity C was found as 7-Epimer of cefixime.

PMR Spectra

The PMR spectra of impurity C in MeOD indicated to a 7-epimer of the compound as given below:

S.No.	Chemical shift (ppm)	Multiplicity	Proton assignment
1.	9.69	d	-CONH-
2.	7.28	b	$-NH_2$
3.	6.8	m	H of Ar
4.	6.72	s	Vinyl
5.	5.52	d	Vinyl
6.	5.27	d	Vinyl
7.	4.95	s	6-H
8.	4.94	d	7-H
9.	4.60	s	$-O-CH_2$
10.	3.62-3.81	q	$-S-CH_2$

The NMR data is similar to cefixime only change is the signal of C-6, and C-7 proton are shifted upfield and no coupling is observed with CONH proton therefore the structure assigned as 7-epimer of cefixime.

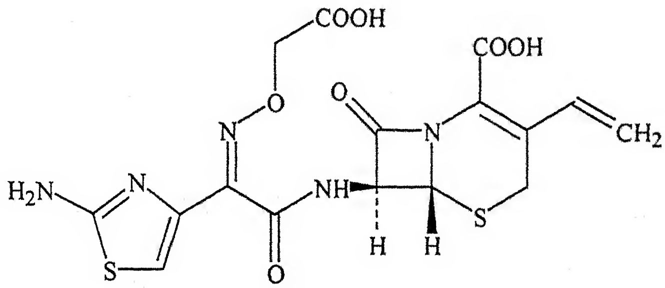
Mass Spectra

The assignment to the molecular ion peaks are given below:

S.N.	m/z amu	Structural
1	476 (M+Na)+	Molecular ion
2	492.1 (M+K)+	Molecular ion
3	454.1 (M+H)+	Molecular ion

Summary

The unidentified impurity C was isolated from Cefixime using preparative HPLC. The structure of this impurity was assigned on the basis of PMR and Mass Spectra. It was identified as 7-epimer of cefixime. (7S)-7-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy)prop-2-enoylamino]-1-aza-8-oxo-5-thia-3-vinylbicyclo[4.2.0]octane-2-carboxylic acid.



Isolation of cefixime impurity E (RRT 0.79)

Analyzing Cefixime initially and after accelerated stability study, it was found that this impurity was not formed, Hence it was decided to synthesize it.

Process for the synthesis of impurity 'E'

Took 7-ADCA (5.0gm) in a 4-neck RBF followed by Dichloromethane (10 times of 7-ADCA). Stirred at RT for 10 min. than added HMDS (0.8M), and TMCS (0.60 M), this reaction mix was heated to reflux for 3 hrs followed by cooling to –10°C. DATMA was added to the silylated mass of 7-ADCA at –10°C in one lot and reaction mass stirred at same temp for 1.0 hrs

The reaction mass was washed thrice with 10% sodium carbonate solution and thereafter organic layer was separated and concentrated under vacuum. The resulted reaction mass was subjected to deprotection in the presence of $AlCl_3$ (2M) and Anisole (10M) and after completion of reaction it was added to the another RBF containing HCl (10M) and water (10 times of 7-ADCA) at 0 - 5°C, the precipitated hydrochloride was basified up to pH 6.5, and finally by adjusting pH 3.5 with HCl 10% pure product was isolated. The purity of the product was found 98% by HPLC (area normalization method).

Conclusion

The structure of impurity synthesized was found as impurity 'E'

PMR Spectra

The PMR spectra in DMSO d6 confirm the structure of impurity 'E'.

S.No.	Chemical shift (ppm)	Multiplicity	Proton assignment
1.	9.51	d	-CONH
2.	7.28	b	$-NH_2$
3.	6.80	s	H of Ar
4.	5.55	q	4-H
5.	5.11	d	6-H
6.	4.59	s	$-O-CH_2$
7.	3.2-3.7	q	$-S-CH_2$
8.	2.2	s	$-CH_3$

The NMR data shows the presence of $-CH_3$ protons at 2.2 ppm and absence of vinyl $-CHCH_2$ protons signal indicate the structure of impurity E.

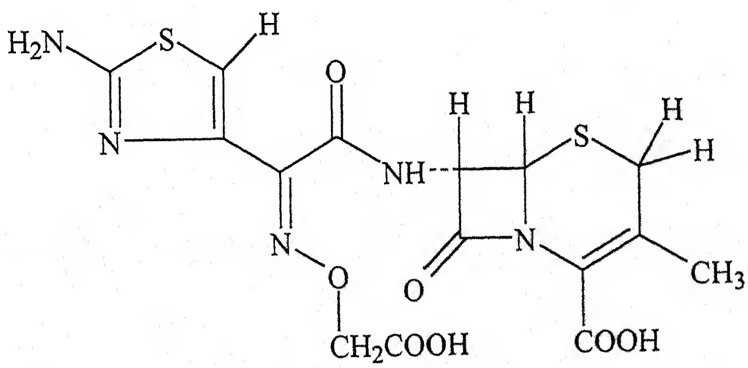
Mass Spectra

The assignment to the molecular ion peaks is given below:

S.N.	m/z amu	Structural
	442.4 (M+H)+	Molecular ion

Summary

As per structure of impurity E, several attempts were made to synthesize the same. Finally it was prepared using 7-ADCA and DATMA. PMR and Mass Spectra confirmed the structure of the impurity. (6R)-6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl)-3-aza-3-(carboxymethoxy)prop-2-enoylamino]-3-methyl-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid



Isolation of impurity F (2.15RRT)

The initial experiments were carried out to study the stability of the impurity on the MPLC system using following mobile phase and gradient system.

Mobile Phase	- 0.1m Ammonium Acetate (Buffer): Methanol
Solution A	- Buffer: Methanol (80 : 20)
Solution B	- Buffer: Methanol (50 : 50)
Wavelength	- 254 nm
pH	- 3.2 (Adjusted with H_3PO_4)
Flow	- 140.0ml/min

Gradient condition

S.N.	Time	Event	Value	Flow ml/min
1	00.01	B.conc	00.0	140.00
2	10.00	B.conc	00.0	140.00
3	25.00	B.conc	80.0	140.00
4	30.00	B.conc	100.0	140.00
5	40.00	B.conc	100.0	140.00
6	50.00	B.conc	00.0	140.00
7	60.00	B.conc	00.0	140.00
8	65.00	B.conc	Stop	140.00

MPLC was employed for initial enrichment of the impurity sample up to about 60.0%. All fractions collected were monitored by the analytical method. The fractions containing impurity were

Concentrated on rotavapour at bath temperature 38-40°C using high vacuum to distill the methanol. The solution then lyophilized, and thereafter loaded on preparative HPLC for desalting and further purification.

For desalting and purification following Prep.HPLC condition employed.

Column	- X-terra (300 * 19.0 mm, 7.0 μm particle)
Mobile Phase	
Solution A	– Water + 1.0ml/Lit. TFA in Mobile Phase
Solution B	– Water: Methanol (1: 1.2 Ratio) +1.0ml TFA /Lit. Mobile Phase
Wavelength	- 254nm
Flow	- 15.0ml/min

Prep.Gradient.

S.N.	Time	Event	Value	Flow ml/min
1	00.01	B.conc	00.0	15.0
2	05.00	B.conc	00.0	15.0
3	25.00	B.conc	80.0	15.0
4	35.00	B.conc	100.0	15.0
5	45.00	B.conc	100.0	15.0
6	50.00	B.conc	00.0	15.0
7	55.00	B.conc	00.0	15.0
8	56.00	B.conc	Stop	15.0

The fractions containing the impurity were collected and lyophilized. The solid mass was taken for Purity evaluation by analytical system and other spectroscopic studies.

Conclusion

The structure of impurity was found as a dimmer of cefixime and its thiazole moiety.

PMR Spectra

The PMR spectra of impurity F in DMSO d6 indicated to a dimeric structure of the compound.

S.NO.	Chemical shift (ppm)	Multiplicity	Proton assignment
1.	13.22	s	-COOH
2.	9.66-9.70	d	-CONH
3.	7.49	s	-NH
4.	7.26	s	$-NH_2$
5.	6.89	d	Vinyl
6.	6.83	s	H of Ar
7.	5.85	dd	7-H
8.	5.55	d	Vinyl
9.	5.29	d	Vinyl
10.	5.21	d	6-H
11	4.62	d	$-OCH_2$
11.	3.80	d	$-S-CH_2$

The NMR signals of δ 7.49 –NH and $[M+H]^+$ at 681.2 It was identified as dimeric impurity of cefixime at Thiazole moiety.

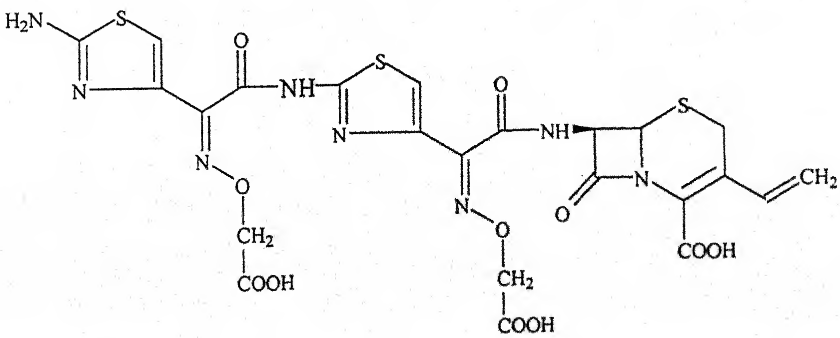
MASS SPECTRA

The assignment to the molecular ion peaks is given below:

S.N.	m/z amu	Structural
1	702.9 (M+Na)+	Molecular ion
	681.2 (M+H)+	Molecular ion

SUMMARY

The unidentified impurity F was isolated from Cefixime using MPLC and preparative HPLC. The structure of this impurity was assigned on the basis of PMR and Mass Spectra. It was identified as dimeric impurity of cefixime and its Thiazole moiety.(6R)-6-((2Z)-2-{2-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy)prop-2-enoylamino](1,3-thiazol-4-yl)}-3-aza-3-(carboxymethoxy)prop-2-enoylamino)-5-oxo-3-vinyl-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid.



Isolation of impurity I (2.52RRT)

Weighed about 1.0gm of Cefixime B.No.CXM/080005 in 25.0ml beaker, add20.0ml water and added Triethyl Amine till sample completely soluble. Load this solution on MPLC using following gradient condition.

Column	- 700mm * 50mm glass column (packed with C-18, 20-40μm particle size)
Buffer	- Water + Acetic acid (0.1%)
Solution A	- Buffer: Acetonitrile (80: 20)
Solution B	- Buffer: Acetonitrile (50: 50)
pH	- As such
Wavelength	- 254nm
Flow	- 100ml/min.

S.N.	Time	Event	Value	Flow ml/min
1	00.01	B.conc	00.0	100.00
2	15.00	B.conc	00.0	100.00
3	25.00	B.conc	40.0	100.00
4	35.00	B.conc	100.0	100.00
5	50.00	B.conc	100.0	100.00
6	55.00	B.conc	00.0	100.00
7	70.00	B.conc	00.0	100.00

MPLC was used for initial enrichment of impurity up to 90%. All the fractions were monitored by analytical system. The fraction containing impurity was concentrated using rotavapour at bath temperature 32-35°C by high vacuum to distill the acetonitrile. The

Aqueous solution was lyophilized and thereafter loaded on prep. HPLC for further purification under following gradient condition.

Column	- Spherisorb Waters C-18 (250mm * 20mm, 10μm particle)
Buffer	- ware + Acetic acid (0.1%)
Solution A	- Buffer: Acetonitrile (80: 20)
Solution B	- Buffer: Acetonitrile (50: 50)
Wavelength	- 254nm
Flow	- 15.0ml/min.
pH	- As such

S.N.	**Time**	**Event**	**Value**	**Flow ml/min**
1	00.01	B.conc	00.0	15.0
2	05.00	B.conc	00.0	15.0
3	25.00	B.conc	60.0	15.0
4	35.00	B.conc	100.0	15.0
5	50.00	B.conc	100.0	15.0
6	55.00	B.conc	00.0	15.0
7	60.00	B.conc	00.0	15.0
	61.00	B.conc	Stop	15.0

The fractions containing impurity were collected and lyophilized. The solid mass was taken for purity evaluation and other spectroscopic studies.

Conclusion

The structure of impurity was found as monoprotected (t-butyl) cefixime.

PMR Spectra

The PMR spectra of impurity I in MeOD confirm the above-mentioned structure.

S.NO.	Chemical shift (ppm)	Multiplicity	Proton assignment
1.	9.6	d	-CONH-
2.	7.3	b	$-NH_2$
3.	7.0	m	Vinyl
4.	6.8	s	H of Ar
5.	5.81	d	7-H
6.	5.5	d	Vinyl
7.	5.25	d	Vinyl
8.	5.10	d	6-H
9.	4.60	s	$-O-CH_2$
10.	3.8	q	$-S-CH_2$
11.	1.60	s	t-butyl

At δ1.60 new proton signals are observed it is because of acetyl group being substituted with t-butyl group. And mass shows $[M+H]^+$ at 510.

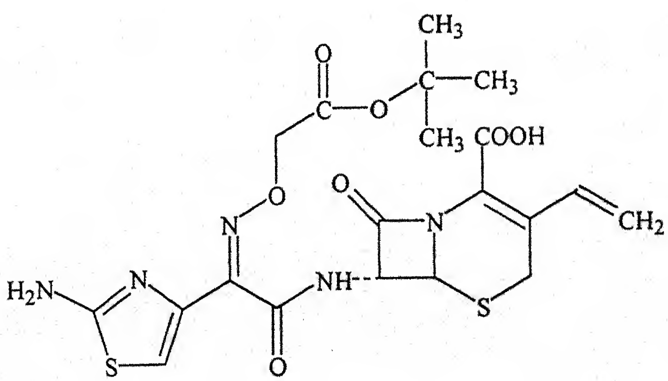
Mass Spectra

The assignment to the molecular ion peaks and their fragments are given below:

S.N.	m/z amu	Structural
1	510 (M+H)+	Molecular ion
2	532 (M+Na)+	Molecular ion

SUMMARY

The unidentified impurity I was isolated from Cefixime using MPLC and preparative HPLC. The structure of this impurity was assined on the basis of PMR and Mass spectra. It was identified as monoprotected (t-butyl) cefixime. (6R)-6-((2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-{[(tert-butyl)oxycarbonyl]methoxy}prop-2-enoylamino)-5-oxo-3-vinyl-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid.



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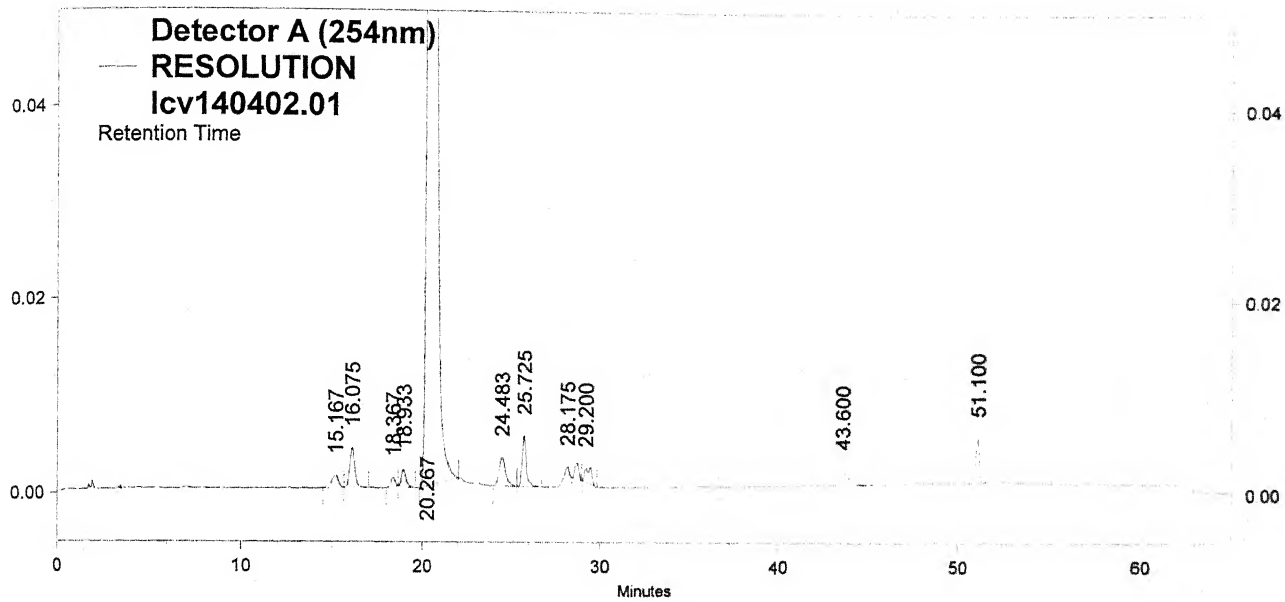

Detector A
(254nm)

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3	18.367 A2	14984	0.22	26714.18	4.85	0.87
4	18.933 A3	29238	0.44	27148.58	1.25	1.23
5	20.267 Cefixime	6152413	91.74	15538.32	2.40	2.26
6	24.483 C	74607	1.11	22336.13	6.46	1.24
7	25.725 D	88014	1.31	60951.73	2.32	1.24
8	28.175 B	73245	1.09	37020.38	4.89	0.80
9	29.200 B1	38732	0.58	54474.50	1.89	2.17
10	43.600 F	52988	0.79	40179.68	21.01	1.74
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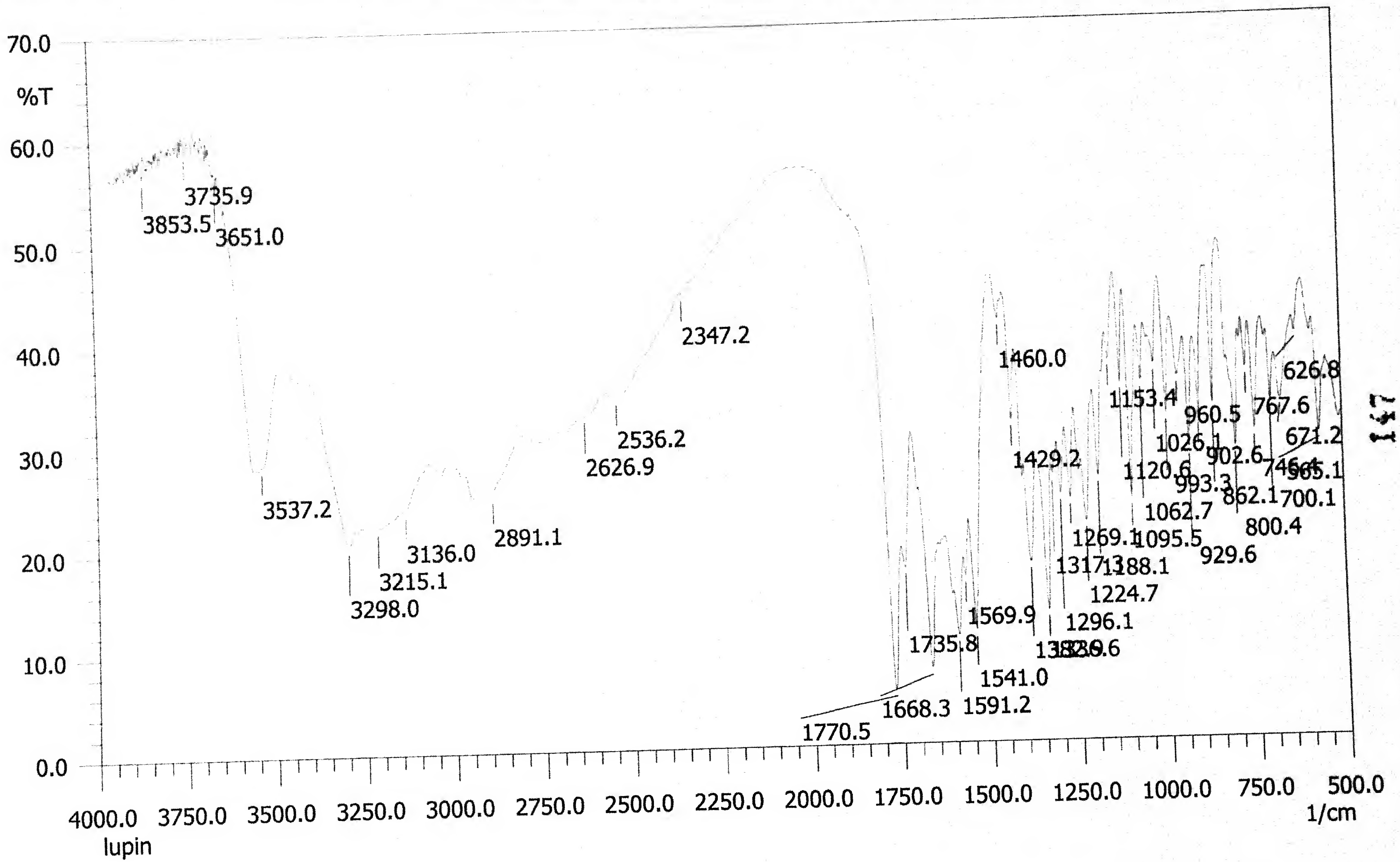
Totals		6706243	100.00			



019STD.IRS: lupin

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Abscissa:	1/cm	Ordinate:	%T	Apodization:	Happ		
Min:	401.17	Max:	3998.16	Range:	1/cm		
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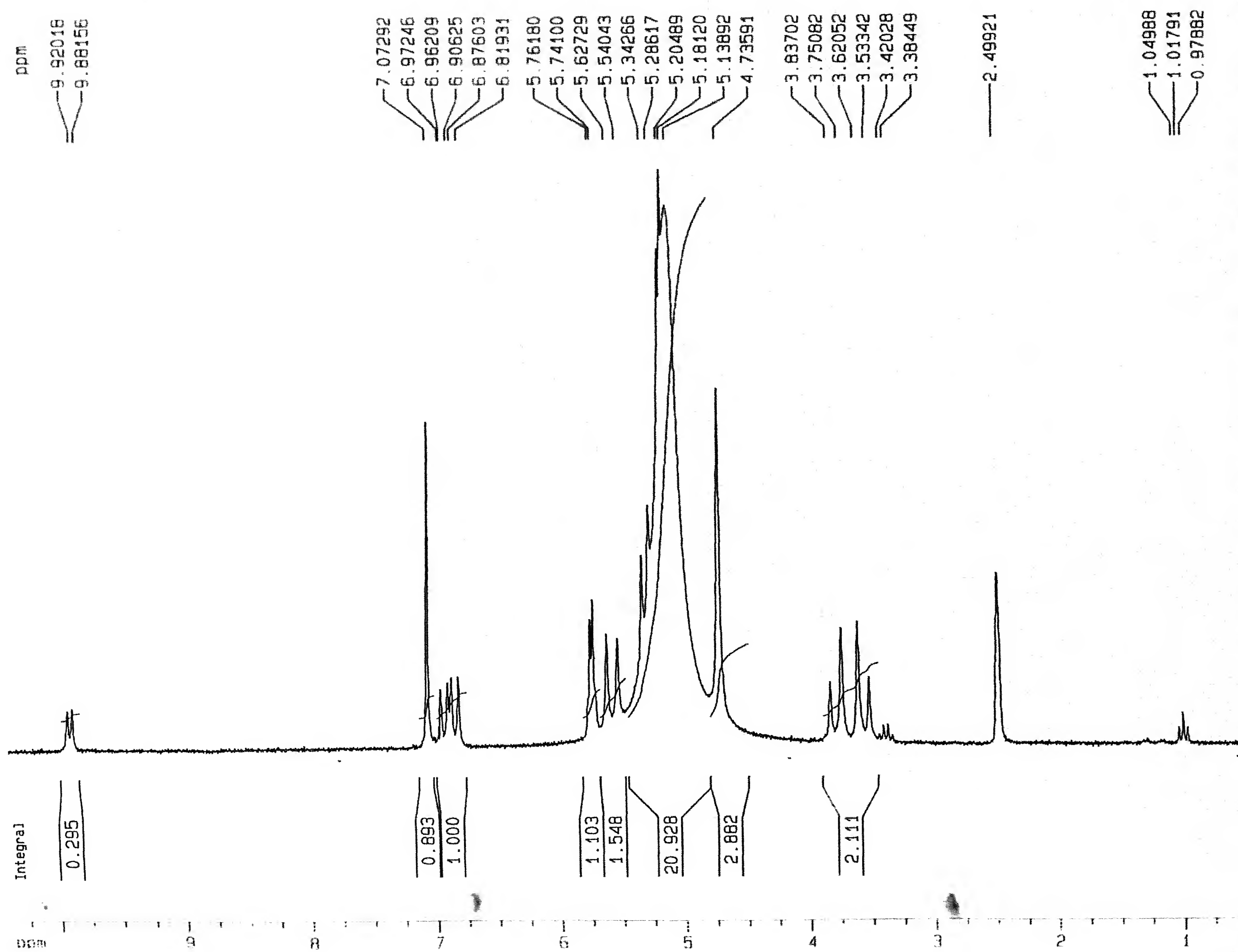
H1-NMR SPECTRUM OF CEFIXIME WORKING STANDARD IN DMSO-D6

SPEC.NO. 02H077



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F1	2079.39 Hz
F2P	0.443 ppm
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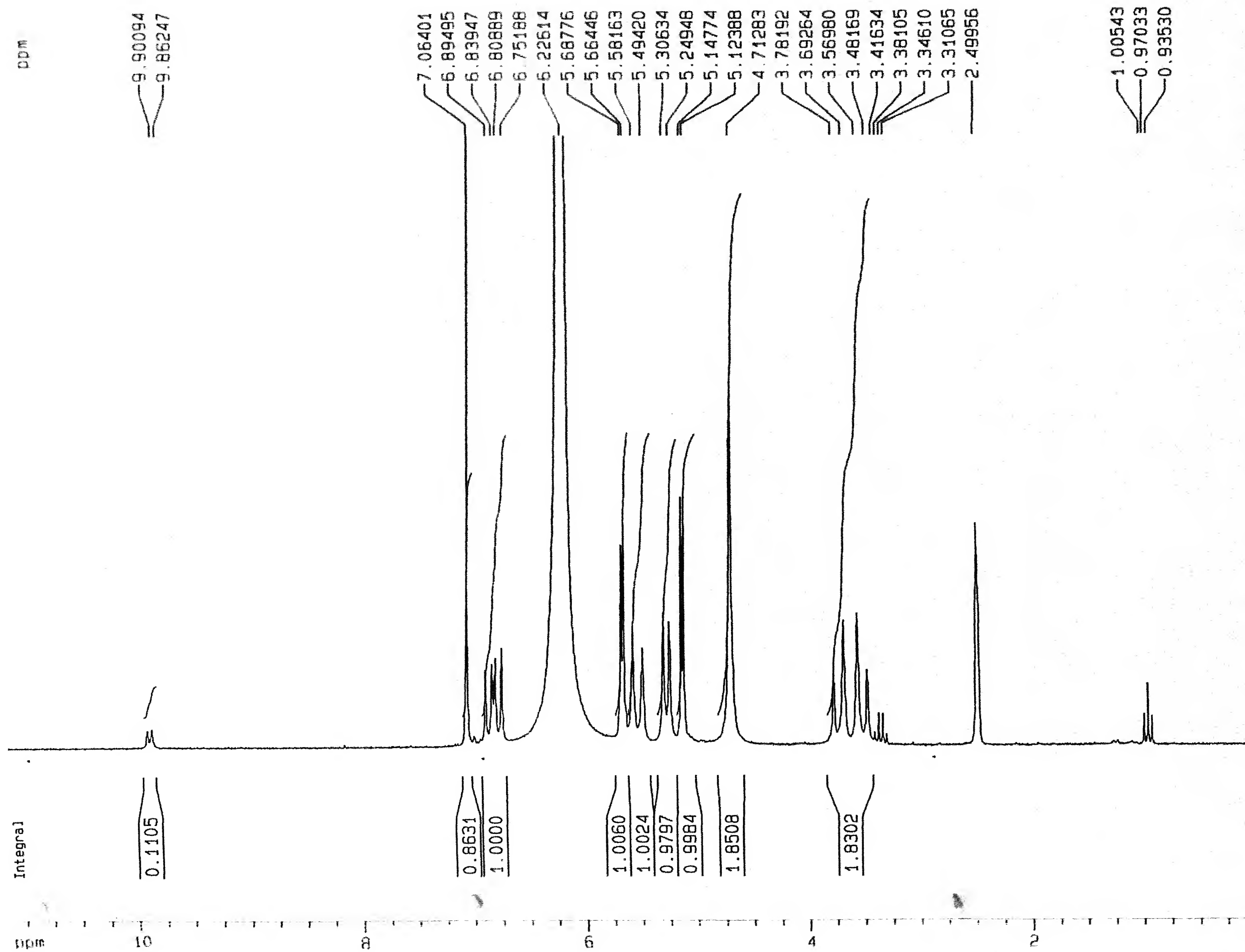
H1-NMR SPECTRUM OF CEFIXIME W.S.IN DMSO-d6+dcL



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FIDRES 0.122266 Hz
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TE 300.0 K
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F2 - Processing parameters
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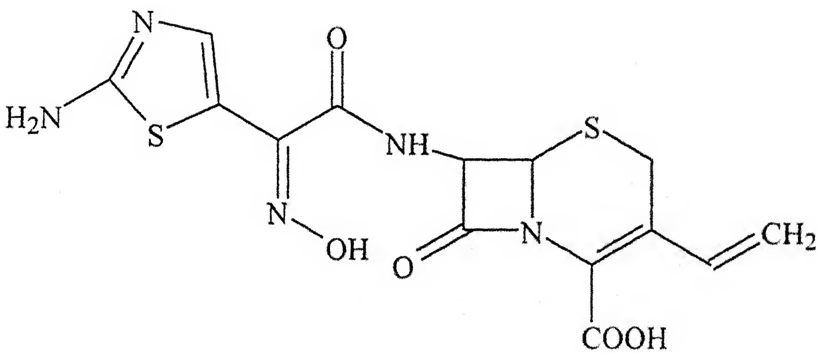
149

CEFDINIR



CEFDINIR

Chemical Names :

- (6R,7R)-7-[[(2Z)-(2-Amino-4-thiazolyl)(hydroxyimino)acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.
- Syn-7-[2-(2-amino-4-thiazolyl)-2-hydroxyiminoacetamido]-3-vinyl-3-cephem-4-carboxylic acid.

Nonproprietary name : Cefdinir

Proprietary Names : Omnicef

Empirical Formulae :

- Free acid $C_{14}H_{13}N_5O_5S_2$

Molecular Weight :

- Free acid 395.42 [$C_{14}H_{13}N_5O_5S_2$]

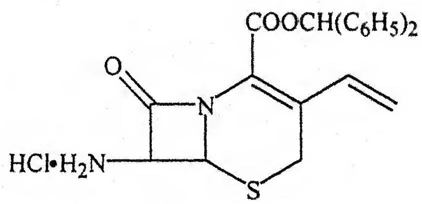
CAS Number : 91832 – 40 –5

Appearance : The cefdinir, which is the article of commerce used in dosage form, is a white to light-yellow crystalline powder. It is odorless, or can have a slight characteristic odor.

Use and Applications : Chemical modification at the C-3 and C-7 positions of the cephem ring has been examined for several decades in order to increase antibacterial activity. Cefdinir, a new orally effective semisynthetic cephalosporin with an extended antibacterial spectrum, has been developed in the New Drug Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. Cefdinir is the first member of what is generally termed the third generation orally active cephalosporins. These third generation cephalosporins are distinct from the older β-lactam antibiotics in their intensive antibacterial activity against a wide range of gram-negative bacteria. The exceptional antibacterial activity of the third generation cephalosporins has been shown to be based on both their enhanced affinity for the target enzymes and their high stability to β-lactamase. Cefdinir shares similar characteristics with other third-generation cephalosporins. Although Cefdinir is less active against staphylococci than are other orally active β-lactam antibiotics, it is far more potent against a wide range of gram-negative bacteria.

The aminothiazole ring appears to be associated with both excellent activity and oral adsorption, and the amino group in the thiazole ring is essential for the potential antibacterial activity. Cefdinir exhibits geometrical isomerism with reference to the configuration of oxime. In the marked contrast to other cephalosporin antibiotics, Cefdinir has a vinyl group at C-3 and a (Z)-2-(2-amino-4-thiazolyl)-2-(hydroxyimino)acetyl moiety at C-7 which influences its improved activity against gram-negative and -positive bacteria[1] and also enhance its pharmacokinetic properties[2] .

Methods of Preparation : Intermediate (5) is common for synthesis of Cefdinir and Cefixime. There is a difference in side chain part only.



(5)

Intermediate –(5)

Side chain synthesis : Stir the suspension of Ethyl-(Z)-2-(2-aminothiazol-4-yl)-2-hydroxyimino acetate in acetonitrile, added triethylamine at control temperature. The mixture was stirred for some time and to this added trityl chloride. The reaction mixture heated at about 60°C for 1.5 hrs. When reaction was complete, acetonitrile was recovered first at atmospheric

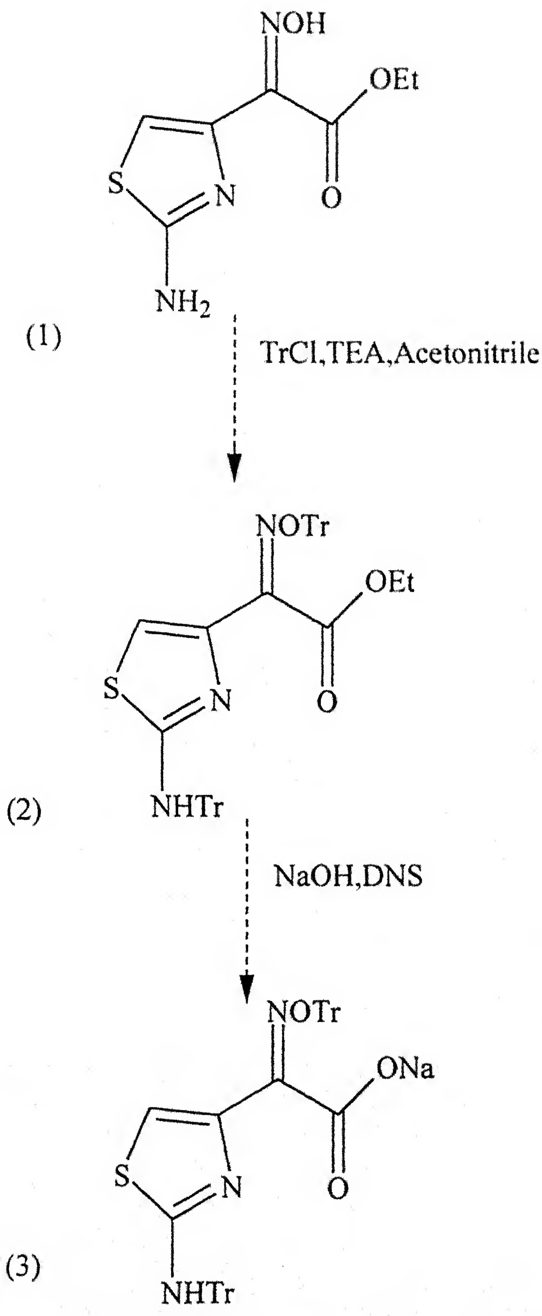
Pressure temperature and then under reduced pressure. To the residue left behind, was added DM water at about 60°C.The resulting slurry was stirred for some time and filtered and washed with DM water. The resulting product is Ethyl-Z)-tritylamino-4-yl-2-trityloxyimino acetate found in good yield (2).

To a solution of sodium hydroxide in ethanol added Ethyl-(Z)-tritylamino-4-yl-2-trityloxyimino acetate at about 60°C and refluxed for 2.0hrs.The progress of reaction was monitored by HPLC. The resultant product was washed with DM water and dried. The resultant product is of (Z)-tritylamino-4-yl-2-trityloxyimino acetic acid sodium salt found in white crystalline solid. (3)

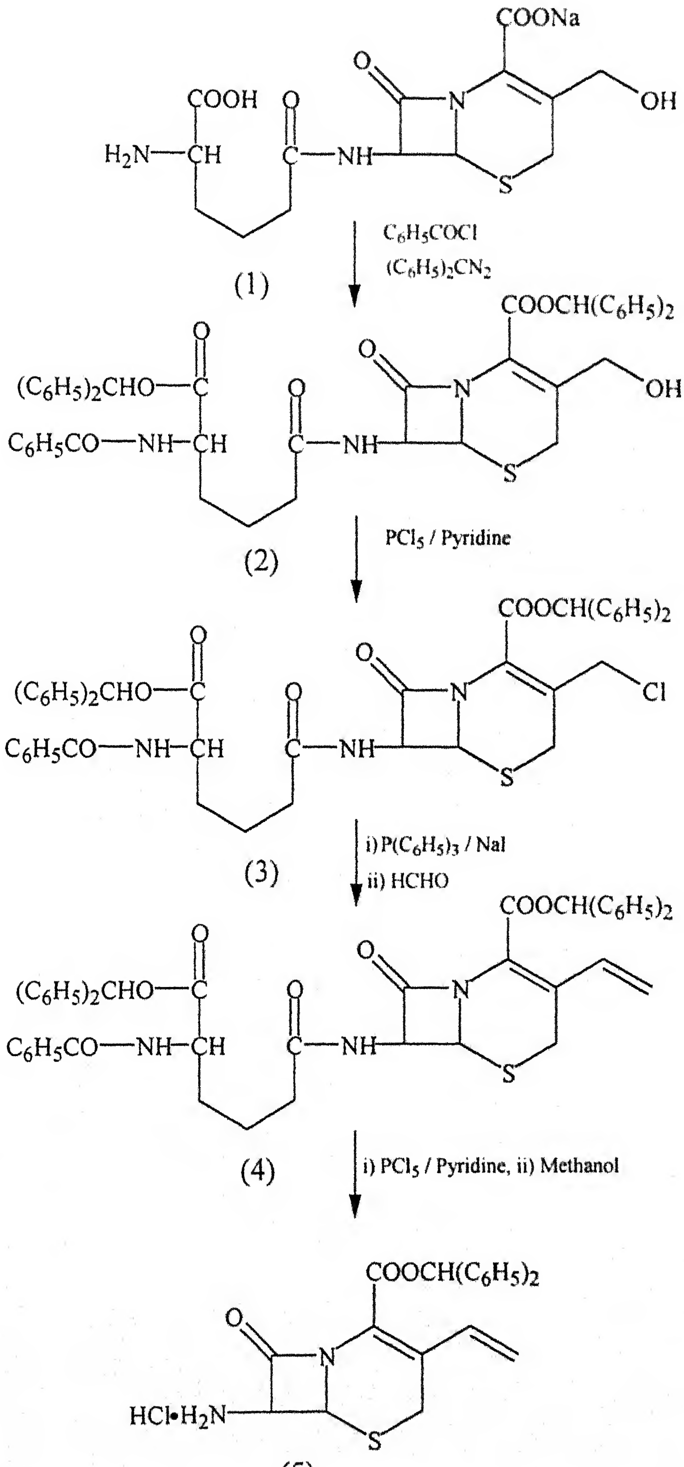
Condensed product: To a solution of (3) in dichloromethane, added intermediate (5). The slurry was stirred for 5.0min.and then Pyridine was added. The reaction mixture was stirred for 10.0min.and then phosphorous oxychloride in DCM was added at control temperature. After stirring adjust the pH of reaction mixture in the range of 2.0 to 3.0 by adding 50% NaOH. The dichloromethane was removed and the residue was diluted with toluene. The DM water was added to toluene layer and it was stirred for 15min. The organic layer was separated washed with water and brine and filtered over a hyflo bed. The resultant organic layer is of 4-methoxybenzyl-7-[2-(2-tritylamino-thiazol-4-yl)-2-(trityloxyimino)acetamido]-3-vinyl-3-cephem-4-carboxylate.(4)

Cefdinir : The organic layer (4) was cooled to 10°C and trifluoroacetic acid was slowly added and stirred for some time at control temperature. Then added DM water, and stirred the mixture further for 30min.Adjust the pH of

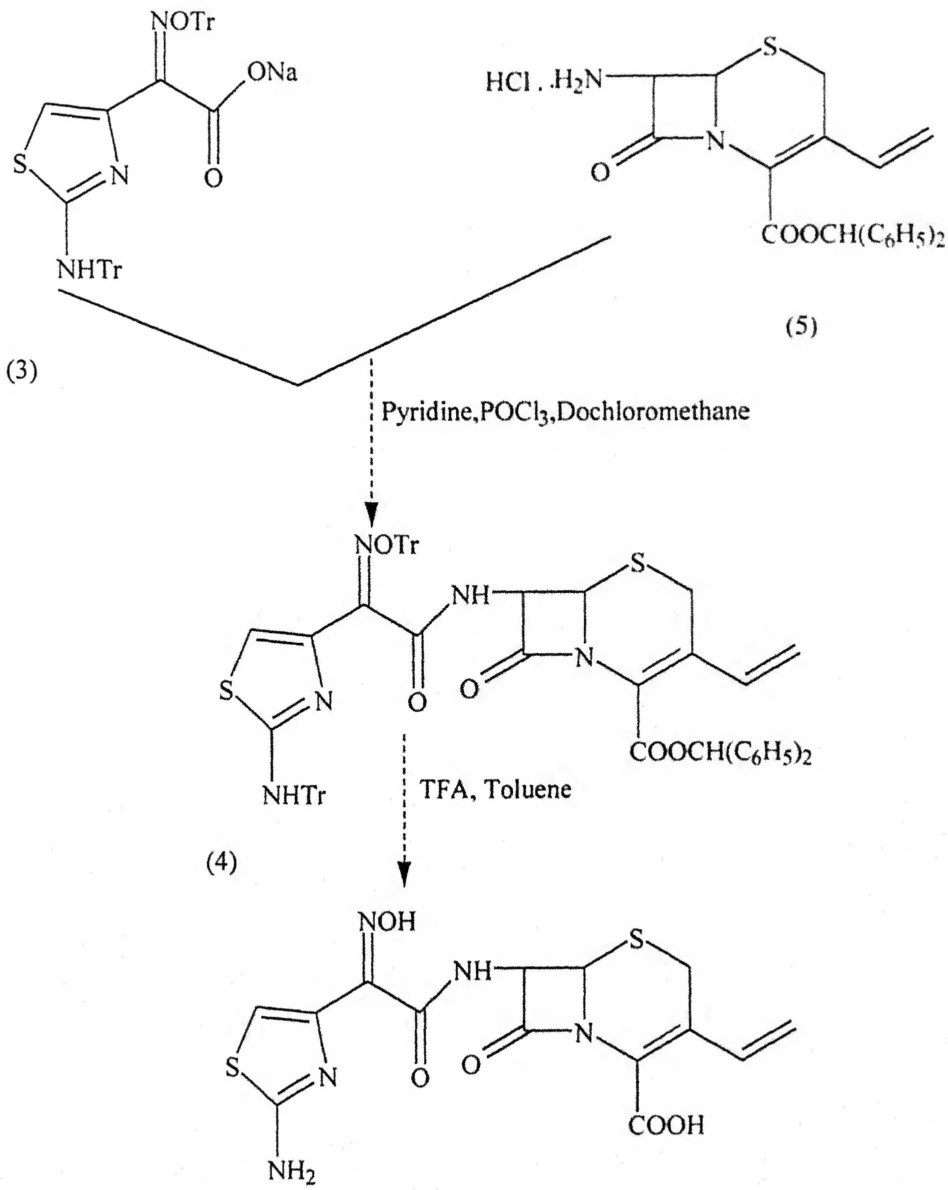
Mixture to 4.5 to 6.0 by aqueous ammonia solution. The aqueous layer thus obtained was cooled and its pH was adjusted to 1.3-1.5 using concentrated HCl. The crystalline solid thus precipitated was washed with DM water and dry to afford Cefdinir as a white crystalline solid.(6).



(Z)-tritylamino-4-yl-2-trityloxyimino acetic acid sodium salt



Synthetic route for the preparation of intermediate 5.



CEFDINIR

(6)

Physical Properties : This property can be checked by Scanning electron photomicrograph and by X-ray powder diffraction pattern.

Optical Rotation : The specific rotation of cefdinir at 25°C in 1.0% aqueous solution of sodium bicarbonate(2.0% concentration of sodium bicarbonate) is between –62° and –70°.

Thermal Analysis : This analysis can be carried out by Melting point apparatus, Differential scanning calorimeter, and Thermogravimetric analysis.

Hygroscopicity : Cefdinir exist in two crystal forms, one is crystal-A and another is crystal-B. Crystal-A contain about 0.2% water, while crystal-B contain about 6.0-9.0% water. When both the form expose to humidity 75.0%, crystal-A absorbed about 7.0% while crystal-B absorbed about 10.0-12.0% water.

Solubility Characteristics : Freely soluble in Dimethyl Sulfoxide and Dimethyl Formamide, slightly soluble in methanol.

Spectroscopy

1. **Vibrational Spectroscopy** : The infrared absorption spectra of cefdinir was obtained using Perkin-Elmer 1650 FT-IR spectrophotometer, where the KBr pellet method of sample preparation was used to prepare the samples.

Assignments for the I.R. of Cefdinir

Energy (cm^{-1})	Band Assignment
3301.9	Symmetric and antisymmetric NH stretches of the carbamate NH_2 group.
2977.9	6-H,7-H stretching modes in the β-lactam ring
1782.1	β-lactam C=O stretching mode
1759	Carbamate C=O stretching mode
1668	Amide I C=O stretching mode
1610	Oxime C=N stretching mode
1332.7,1348	Carbamate NH_2 bending mode
1049, 1014	C-O and N-O stretches of the carbamate and oxime moieties in the CH_2O groups

2. **NMR:** ^1H – Spectrum :

The ^1H-NMR spectra of cefdinir was obtained using 200 MHz Bruker Instrument model DRX-200 NMR spectrometer. The data was obtained at ambient temperature in DMSO-d6, at a concentration of 10 mg/ml. The nomenclature of the assignments is based on the following alphabetical designation:

Chemical Shift	Multiplicity	Relative number of protons
11.33	Singlet	1
9.5	Doublet	1
7.15	Singlet	2
7.00-6.88	Multiplet	1
6.69	Singlet	1
5.84-5.77	Quartet	1
5.61	Doublet	1
5.33	Doublet	1
5.21	Doublet	1
3.90-3.53	Quartet	2

3. **Mass Spectrometry :** The mass spectra of cefdinir was obtained using a PE Biosystem API-3000 instrument, The spectrum of cefdinir is characterized by the presence of an intense protonated molecular ion at m/z 396 (MH^+).

REFERENCES

1. (a) sakane, K.; Inamoto, Y.; Takaya, T. Jpn. Antibiot.1992, 45, 909-925. (b) Sakane, K.; Kawabata, K.; Inamoto, Y.; manaka, H.; Takaya, T. Yakugaku Zasshi 1993, 113, 605-626. (c) Soejima, R. Jpn.J. Antibiot.1992,45,1239-1252. (d) Palmen,D. Structure-Activity Relationships Among the semisynthetic Antibiotics; Acedemic Press; New York, 1947;pp 87-237. (e) Mine, Y.; Kamimura, T.; Sakamoto, H.; Tawara, S.; Hatano, K.; Watanabe, Y.; Kuwahara, S. Chemotherapy 1989, 37,(S-) 100-121.
2. (a) Mine, Y.; Watanabe, Y.; Matsumoto, Y.; Kuno,K.; Hatano, H.; Higashi, Y.; Kuwahara, S. Chemotherapy 1989, 37,)S-2) 122-134. (b) Shimada, K.; Shishido, R.; Kakuno, K. Chemotherapy 1989, 37, (S-2) 208-245.

Validation of Cefdinir Assay Method

VALIDATION CRITERIA

	TEST PERFORMED	LIMITS
1.	**Specificity**	Resolution not less than 4.0 Tailing factor 2.0 (max.) Theoretical plate not less than 2000
2.	**Accuracy**	Average % Recovery 100 ± 1.5 %
3.	**Precision**	
	(I) Repeatability	RSD of Area NMT 1.0% RSD of RT NMT 1.5%
	(II) Intermediate Precision	RSD of Area NMT 1.0%RSD RSD of RT NMT 1.5%
	(III) Reproducibility	Analytical error allowed up to 1.0%
4.	**Linearity**	Coefficient of correlation = 0.999
5.	**Robustness**	Resolution not less than 4.0 Tailing factor 2.0 (max.) Theoretical plate not less than 2000
6.	**Degradation study**	Cefdinir peak should not be contaminated by other impurity Confirming by spectrum correlation and contour plot
7.	**Solution stability**	Peak area difference from initial value should be not less than 1.0%

CEFDINIR

Method Validation For The Determination Of Assay

OBJECTIVE: To ensure the validity of analytical procedure for the determination of Assay.

SCOPE: The analytical method is validated as per ICH Guidelines Q2 A & B for the following parameters :-

1. Specificity
2. Linearity
3. Accuracy
4. Precision
5. Robustness
6. Degradation study
7. Solution Stability

HPLC CONDITIONS FOR ASSAY

Preparation of Mobile Phase :

12.474gm of Citric Acid was dissolved in 1800 ml of distilled water adjustd the pH-2 by H_3PO_4. Add 37.5ml of Methanol & 100ml 1-4 Dioxane, filtered the mixture through 0.2µ membrane.

Solvent: (For Dilution Purpose) Mobile phase was used for dilution purpose.

Name of Equipment

Instrument	Shimadzu LC-10AVP series
System No.	15,16 22
HPLC Pump	Shimadzu LC-10ATVP
HPLC Detector	Shimadzu SPD – M10AVP &SPD-10AVP
Auto Injector	SIL-10ATVP
System Controller	SCL-10AVP
Column Oven	CTO-10ASVP
Integrator	Computer P-III with Class-VP software ver 5.03
Balance	03

Chromatographic Parameters

Column	Inertsil ODS-3V (4.6 X 150mm), 5µ S.No. OEI83044
Mobile Phase	0.033M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4:MeOH:1,4-Dioxane
Composition	36:0.75:2
pH	2.00 (As such)
Flow Rate	1.5 ml / min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 µl

SPECIFICITY OF CEDINIR

To check the specificity or selectivity for HPLC Assay method of Cefdinir.

Standard preparation:-

Weigh accurately 0.1006 gm of sample B.No. 025/VII/DS/DM-1 in 100ml volumetric flask, dissolve and dilute to the mark with dilution buffer & pipette out 10ml of this solution into 50ml of volumetric flask make up to the mark with mobile phase.

Observation:-

S.No.	Sample Name	File Name	R.T.	Theoretical Plates	Tailing Factor	Resolution
1.	Impurity	LC050101.05	9.03	--	--	
2.	Cefdinir	LC050101.05	14.20	2503	1.07	9.10

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion:

- Theoretical plate ,Tailing Factor and Resolution were within the acceptable limit.

RECOVERY OF CEFDINIR

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy was established across 50 to 150 percent of the test concentration.

Standard Solution(A)–Weighed 0.0503 gm of Cefdinir B.No. 025/VII/DS/DM-1 in 100ml volumetric flask dissolved & diluted to the mark with dilution buffer.

Test Solution(B)– Weighed 0.0503 gm of Cefdinir B.No. 025/VII/DS/DM-3 in 100ml volumetric flask dissolved & diluted to the mark with dilution buffer.

50.3 PPM:- Pipetted 5ml of A solution + 0 ml of B in 50ml volumetric flask & diluted to the mark with Mobile phase.

50.3 PPM:- Pipetted 0ml of B solution + 5 ml of A in 50ml volumetric flask & diluted to the mark with Mobile phase.

100.6 PPM:- Pipetted 5ml of B solution + 5 ml of A in 50ml volumetric flask & diluted to the mark with Mobile phase.

201.2 PPM :- Pipetted 5ml of B solution + 15 ml of A in 50ml volumetric flask & diluted to the mark with Mobile phase.

301.8 PPM:- Pipetted 5ml of B solution + 25 ml of A in 50ml volumetric flask & diluted to the mark with Mobile phase.

S.No.	Conc. (PPM)	Theoretical Area	Practical Area	Recovery	File Name
1.	50.3 (A)	-	1384008.33	--	CE0712.02-04
2.	50.3 (B)	-	1394902.33	--	CE0712.05-07
3.	100.6	2778910.66	2777623.66	99.95%	CE0712.08-13
4.	201.2	5546927.32	5550213.50	100.06%	CE0712.14-19
5.	301.8	8314943.98	8297439.66	99.79%	CE0712.20-25

**All above solutions were injected for three times to calculate % Recovery.

Average recovery - 99.93%

Remark- Average recovery were found within the acceptable limit.

PRECISION OF CEFDINIR

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the specified conditions.

Following determinations are carried out for establishing the Precision of Assay method:

- Repeatability
- Intermediate Precision (Ruggedness)
- Reproducibility

Repeatability

Standard preparation : (A) : Weighed 0.0503gm of Cefdinir B.No. 025/VII/DS/DM-1 in 100ml volumetric flask, dissolved and diluted to the mark with dilution buffer. Solution concentration was 50.3 PPM.

Test preparation : (B) : Weighed 0.0503gm of Cefdinir B.No. 025/VII/DS/DM-3 in 100ml volumetric flask, dissolved and diluted to the mark with dilution buffer. Solution concentration was 50.3 PPM.

$$\text{Standard Deviation} = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{(n-1)}}$$

$$\text{Relative Standard Deviation} = \frac{(\text{Standard Deviation} \times 100)}{\text{Average}(\bar{X})}$$

100.6 PPM:- Pipetted 5ml of solution B and 5ml of solution A to 50ml volumetric flask & diluted to the mark with Mobile phase.

201.2 PPM :- Pipetted 5ml of solution B and 15ml of solution A to 50ml volumetric flask & diluted to the mark with Mobile phase.

301.8 PPM :- Pipetted 5ml of solution B and 25ml of solution A to 50ml volumetric flask & diluted to the mark with Mobile phase.

S.No.	Conc. (PPM)	Avg. Area	RSD of Area	Criteria	RSD of RT	Criteria	File Name
1.	100.6	2777623.66	0.29%	RSD of Area= NMT 1.0%	0.001%	RSD of RT= NMT 1.5%	CE0712.08-13
2.	201.2	5550213.50	0.14%		0.05%		CE0712.14-19
3.	301.8	8297439.66	0.07%		0.03%		CE0712.20-25

**All the three solutions were injected for six times for calculating RSD of RT& Area.

Conclusion : RSD of Retention time & Area were found within the acceptable limit in a range from 100.6PPM to 301.8 PPM.

Intermediate Precision

A matrix of experiment is designed to include random circumstantial events on Precision of an analytical method. Hence following changes are made :-

- Analyst
- Instrument
- Inter day variation

Sample preparation :
Weighed 0.1010gm of Cefdinir B. No. 025/VII/DS-98 in 100ml volumetric flask, dissolved and diluted to the mark with dilution buffer. Pipetted 10ml of this solution and diluted to 50ml with mobile phase. Solution concentration was 202PPM.

Sample preparation :
Weighed 0.1012gm of Cefdinir B. No. 025/VII/DS-98 in 100ml volumetric flask, dissolved and diluted to the mark with dilution buffer. Pipetted 10ml of this solution and diluted to 50ml with mobile phase. Solution concentration was 202.4PPM.

Sample preparation : Weighed 0.1004gm of Cefdinir B.No. 025/VII/DS-98 in 100ml volumetric flask, dissolved and diluted to the mark with dilution buffer. Pipetted 10ml of this solution and diluted to 50ml with mobile phase. Solution concentration was 200.8PPM.

$$\text{Standard Deviation} = \sqrt{\frac{\sum_{i=1}^{n} (Xi - \bar{X})^2}{(n-1)}}$$

$$\text{Relative Standard Deviation} = \frac{(\text{Standard Deviation} \times 100)}{\text{Average}(\bar{X})}$$

S.No.	Conc. (PPM)	Avg. Area	RSD of Area	Criteria	RSD of RT	Criteria	Analyst
1.	202.0	5582351.8	0.07%	1.0%	1.26%	1.5%	Analyst-1
2.	202.4	5634039.0	0.13%	1.0%	1.33%	1.5%	Analyst-2
3.	200.8	6140790.6	0.101%	1.0%	0.424%	1.5%	Analyst-3

**All above solutions were injected for six times to calculate the RSD of RT & Area.

Conclusion : RSD of Retention time & Area were found within the acceptable limit.

Reproducibility

To check the variation in assay of Cefdinir using different analysts.

Standard Preparation : Weigh accurately about 0.1000gm of standard sample B.No.025/VII/DS/-98 in 100.0 ml volumetric flask, dissolve and dilute to the mark with dilution buffer. Pipette out 10.0ml of this solution and dilute it to 50.0ml with dilution buffer.

Test Preparation : Weigh accurately about 0.1000gm of test sample in 100.0ml volumetric flask, dissolve and dilute to the mark with dilution buffer. Pipette out 10.0mlof this solution and dilute it to 50.0ml with dilution buffer.

Calculation :-

$$\% \text{ Test purity} = \frac{\text{Area of test}}{\text{Area of std.}} \times \frac{\text{Weight of std.}}{\text{Weight of test}} \times \text{Purity of std.}$$

Calculated Assays :

B.No. : 025/VII/DS/DM-3

Analyst	**ANALYSED ON**	**SYSTEM USED**	**AS IS PURITY**	**Max. Variation**	**Criteria**
Analyst-1	12th Sept.2000	System No.15	88.79%	0.92%	NMT 1.0%
Analyst-2	13th Sept.2000	System No.16	89.71%		
Analyst-3	19th Sept.2000	System No.15	89.31%		

B.No. : 025/VII/DS/DM-1

Analyst	ANALYSED ON	SYSTEM USED	AS IS PURITY	Max. Variation	Criteria
Analyst-1	12th Sept.2000	System No.15	88.01%	0.61%	NMT 1.0%
Analyst-2	13th Sept.2000	System No.16	88.62%		
Analyst-3	19th Sept.2000	System No.15	88.13%		

B.No. : 025/VII/DS/89

Analyst	ANALYSED ON	SYSTEM USED	AS IS PURITY	Max. Variation	Criteria
Analyst-1	12th Sept.2000	System No.15	88.96%	0.54%	NMT 1.0%
Analyst-2	13th Sept.2000	System No.16	89.50%		
Analyst-3	19th Sept.2000	System No.15	89.39%		

Conclusion : Variation in assay from analyst to analyst and from system to system were within acceptable limit.

LINEARITY OF CEFDINIR

The linearity of an analytical procedure is its ability (within a given range) to obtain the test results which are directly proportional to the concentration of an analyte in the sample.

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, and accuracy.

Solution Preparations :-

Stock solution- 0.2502gm of Cefdinir B.No. 025/VII/DS/DM-3 was dissolved & diluted to 250ml with dilution buffer. The concentration of this solution was 1000.8 PPM.

100.08 PPM :- 5ml of stock solution was diluted to 50ml with mobile phase.

150.12 PPM :- 5ml of 300 PPM solution was diluted to 10ml with mobile phase.

200.16 PPM :- 5ml of stock solution was diluted to 25ml with mobile phase.

250.20 PPM :- 25ml of stock solution was diluted to 100ml with mobile phase.

300.24 PPM :- 15ml of stock solution was diluted to 50ml with mobile phase.

400.32 PPM :- 10ml of stock solution was diluted to 25ml with mobile phase.

S.No.	Cocn.(PPM)	Average Area	File Name
1.	100.08	2873592	FE0209.06-08
2.	150.12	4343108	FE0209.09-11
3.	200.16	5686056	FE0209.01-14
4.	250.20	7164605	FE0209.15-17
5.	300.24	8660950	FE0209.18-20
6.	400.32	11495339	FE0209.21-23

Observation :

Component Name	Coefficient of correlation	Criteria
CEFDINIR	0.9999	NLT 0.999

Conclusion :

- Graph plotted between Area vs Concentration of Cefdinir was found linear for the range 100.0 to 400.0 PPM.

- Coefficient of correlation is also within the acceptable range.

ROBUSTNESS OF CEFDINIR

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during normal usage.

Typical variations are :-

⇒ Influence of variations on flow rate.

⇒ Influence of variations in buffer strength

⇒ Influence of variations of pH in mobile phase

⇒ Influence of variations in mobile phase composition

Sample preparation:-

Weighed 0.1000gm Cefdinir B.No.025/VII/DS/DM-3 in 100ml volumetric flask dissolved & diluted to the mark with dilution buffer. Pipette out 10ml of this solution and dilute it to 50ml with mobile phase. This solution is used for entire Robustness study.

HPLC CONDITION :-

Column	INERTSIL – ODS (4.6x 150 mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2
pH	2.00
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters were checked.

Observation:-

S.No.	ample ame	File Name	R.T.	Theoretical Plates	Tailing Factor	Resolution
1.	mpurity	LC050101.05	9.03	--	--	
2.	efdinir	LC050101.05	14.20	2503	1.07	9.10

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion:

- Theoretical plate ,Tailing Factor and Resolution were within the acceptable limit.

Changed the Flow Rate by +0.2ml

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2
pH	2.0
Flow	1.7ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters were checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Imp.	8.3	--	--		
Cefdinir	13.19	2686	1.05	9.61	NM1211.04

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion: Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Flow Rate by -0.2ml

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2
pH	2.0
Flow	1.3ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters were checked .

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Imp.	10.808	--			
Cefdinir	17.175	3042	1.16	10.22	NM1211.02

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Strength of buffer salt in mobile phase by +5.0%

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5µ
Mobile Phase	0.0347 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2
pH	2.00
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	9.5				
Cefdinir	15.2	2401	0.88	9.2	FE2209.03

Criteria :

Sample Name	Theoretical Plates	Factor Asymmetry	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed the Strength of buffer salt in mobile phase by -5.0%

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.0314 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2
pH	2.00
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	8.9				
Cefdinir	14.05	2410	0.91	9.05	FE2209.04

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed pH of mobile phase by + 0.5 units

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2.5 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2
pH	2.5
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R. T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	7.86				
Cefdinir	11.79	2402	0.96	8.18	FE2209.02

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed pH of mobile phase by - 0.5 units

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 1.5 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2
pH	1.5
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R. T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	12.8				
Cefdinir	21.8	2593	1.09	7.00	FE2209.001

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed Organic Mobile phase composition by +0.05ml i.e MeOH

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4: MeOH : 1,4 - Dioxane
Composition	36 : 0.80 : 2
pH	2.00
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	8.46				
Cefdinir	13.17	3113	1.15	5.81	ST1411.06

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed Organic Mobile phase composition by -0.05ml i.e MeOH

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5µ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.70 : 2
pH	2.0
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 µl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	8.37				
Cefdinir	13.03	3079	1.16	5.81	ST1411.09

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within Specified limit.

Changed Organic Mobile phase composition by +0.2ml i.e 1,4-Dioxane

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 2.2
pH	2.0
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	7.6				
Cefdinir	11.60	2917	1.16	5.51	ST1411.13

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

Changed Organic Mobile phase composition by - 0.2 ml i.e. 1,4-Dioxane

HPLC CONDITION :-

Column	INERTSIL - ODS (4.6 x 150mm), 5μ
Mobile Phase	0.033 M Citric acid in D.W. and pH adjusted to 2 by H_3PO_4 : MeOH : 1,4 - Dioxane
Composition	36 : 0.75 : 1.8
pH	2.0
Flow	1.5ml/min.
Wavelength	254 nm
Aux. Range	2.0
Injection Volume	20 μl

Sample was injected & system suitability parameters are checked.

Observation :

Sample Name	R.T.	Theoretical Plates	Tailing Factor	Resolution	File No.
Impurity	9.57				
Cefdinir	15.20	3173	1.15	6.46	ST1411.11

Criteria :

Sample Name	Theoretical Plates	Tailing Factor	Resolution
Cefdinir	2000	NMT 2.0	NLT 4.0

Conclusion : Theoretical Plates, Tailing factor & resolution were within specified limit.

DEGRADATION OF CEFDINIR

To establish the validity of assay method for cefdinir subjected to stress conditions of storage.

Experimental Condition : Degradation of Cefdinir was checked under the following stressed storage conditions.

(a) Mobile Phase condition(at 25°c)

(b) Exposure to Photochemical condition (Solid & Solution)

(c) Acidic condition (1N Hydrochloric Acid)

(d) Alkaline condition (1N Sodium Hydroxide)

(e) Oxidative condition (20.0% Hydrogen Peroxide)

(f) Thermal condition (About 50°C in water bath)

Cefdinir B.No.025/VII/DS/DM-3 was taken for the study purpose.

Following are the criteria for conclusion :

Spectrum : Correlate the spectrum of Cefdinir initially and after the experimental condition. The spectrum correlation value more than 99% indicates that no other peak merge with Cefdinir peak.

Contour Plot : Contour plots were taken out initially and at the end of experiment. Cefdinir main peak having similar nucleus, indicates that Cefdinir main peak is pure and not contaminated with any degraded product formed during stressed conditions.

Degradation study in Mobile phase

To check the degradation of Cefdinir in mobile phase.

Solution preparation :

Stock solution : 0.2510gm of Cefdinir was weighed and dissolved and diluted to 250ml with dilution buffer.

Initial solution : 10ml from stock solution was pipetted out immediately after preparation & diluted to 50ml with mobile phase.

Subsequent solutions: Injected initial solution at one hour interval up to 8hrs, then 12 hrs, 24hrs and 48 hrs.

S.No.	Time	Area	% Variance	File Name
01.	Initial	5769007		FE1509.02
02.	After 1Hrs.	5763740	0.09	FE1509.03
03.	After 2 Hrs.	5765022	0.07	FE1509.04
04.	After 3 Hrs.	5764707	0.07	FE1509.05
05.	After 4 Hrs.	5750136	0.33	FE1509.06
06.	After 5 Hrs.	5746918	0.38	FE1509.07
07.	After 6 Hrs.	5738005	0.54	FE1509.08
08.	After 7 Hrs.	5738560	0.53	FE1509.09
09.	After 8 Hrs.	5730234	0.67	FE1509.10
10.	After12 Hrs.	5722023	0.81	FE1509.11a
11.	After24 Hrs.	5706148	1.09	FE1609.01
12.	After 48Hrs.	5635101	2.32	FE1609.13

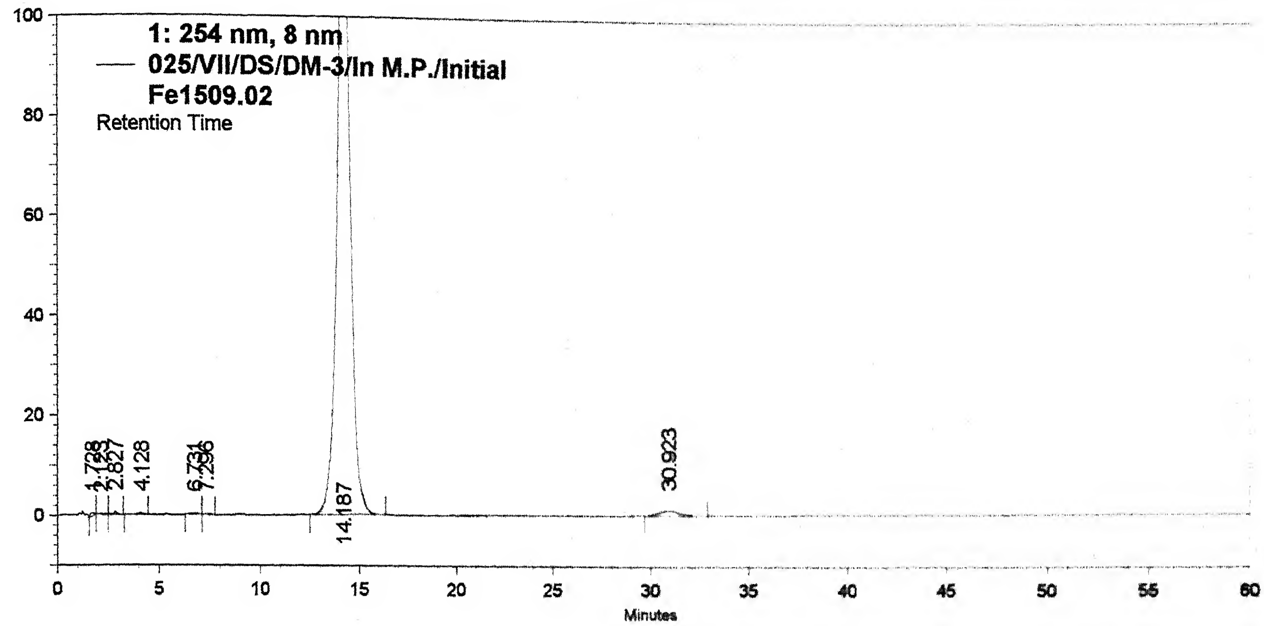
Conclusion :

(1) Spectrum correlation of Cefdinir peak at initial & after 48hrs shows correlation more than 99%. Which confirms that no other peak is merging with Cefdinir.

(2) Contour plot of Cefdinir peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefdinir.

(3) Solution was stable up to 12 hours as per limit of validation criteria.

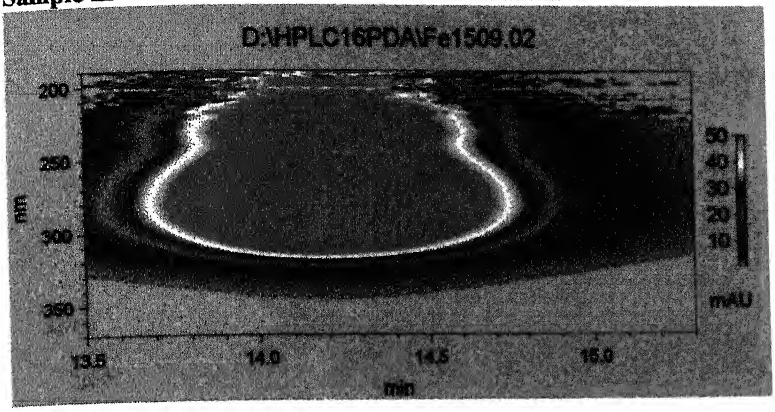
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1509.02
Acquired Time : 9/15/00 8:47:37 AM
Sample ID : 025/VII/DS/DM-3/In M.P./Initial



1: 254 nm, 8 nm

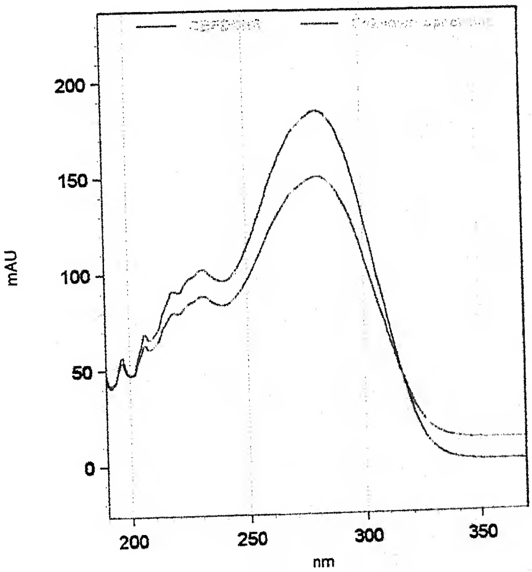
Pk #	Retention Time	Area	Area Percent	Name
1	1.728	8476	0.14	
2	2.123	2033	0.03	
3	2.827	5865	0.10	
4	4.128	5615	0.10	
5	6.731	4868	0.08	
6	7.296	1420	0.02	
7	14.187	5757106	98.30	CEFDINIR
8	30.923	71149	1.21	
Totals		5856532	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1509.02
Acquired Time : 9/15/00 8:47:37 AM
Sample ID : 025/VII/DS/DM-3/In M.P./Initial



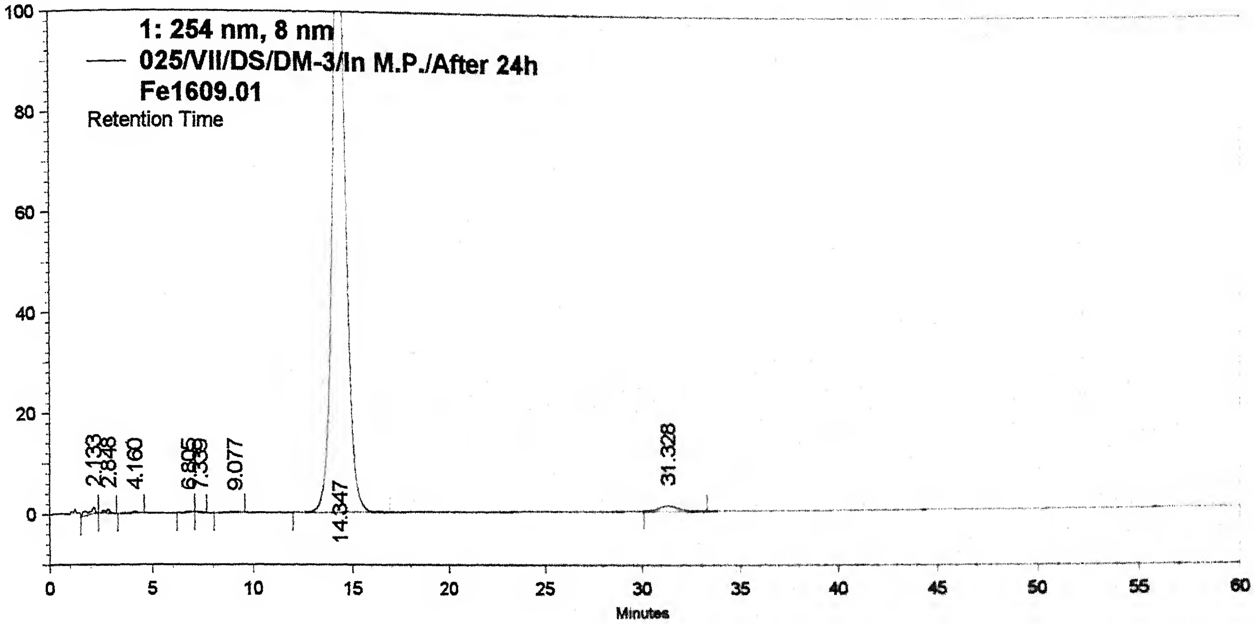
Peak Name: CEFDINIR

Number of Hits: 1



Hit 1
Name: CEFDINIR
Similarity Index: 1.000000
Library: C:\CLASS-VP\Spec\CFDmp.lib

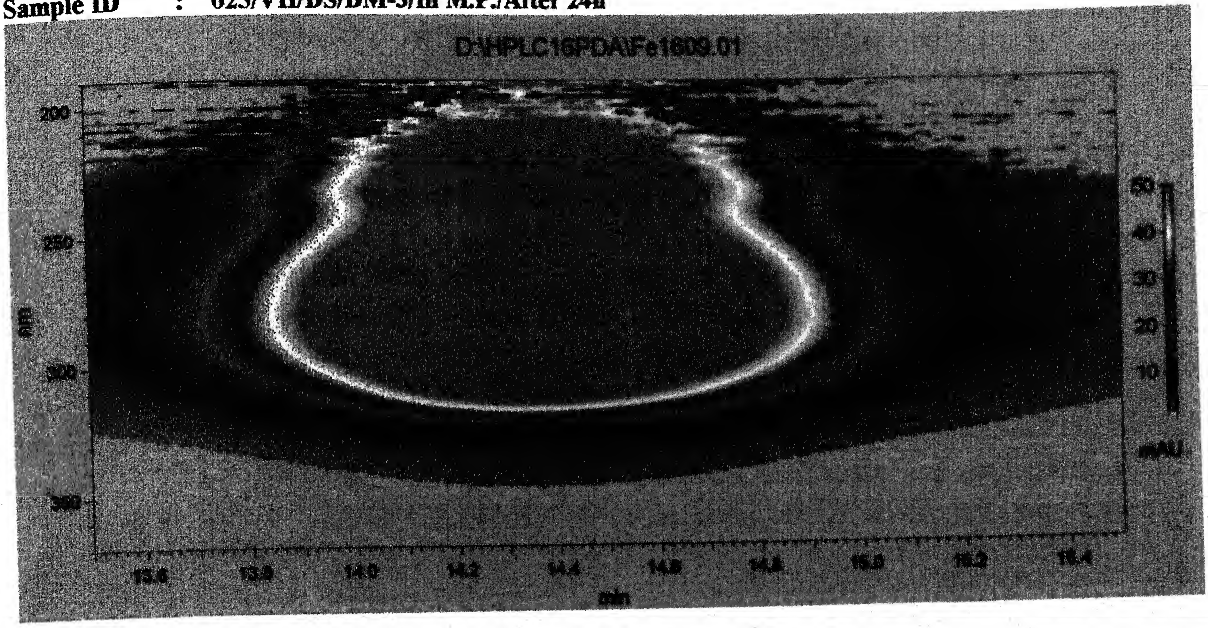
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1609.01
Acquired Time : 9/16/00 8:47:25 AM
Sample ID : 025/VII/DS/DM-3/In M.P./After 24h



1: 254 nm, 8 nm

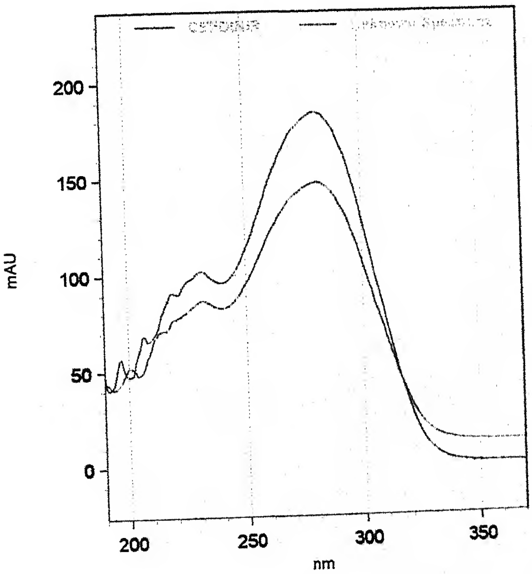
Pk #	Retention Time	Area	Area Percent	Name
1	2.133	32399	0.55	
2	2.848	14005	0.24	
3	4.160	7112	0.12	
4	6.805	4691	0.08	
5	7.339	2662	0.05	
6	9.077	7247	0.12	
7	14.347	5703379	97.60	CEFDINIR
8	31.328	72056	1.23	
Totals		5843551	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1609.01
Acquired Time : 9/16/00 8:47:25 AM
Sample ID : 025/VII/DS/DM-3/In M.P./After 24h



Peak Name: CEFDINIR

Number of Hits: 1



Hit 1
Name: CEFDINIR
Similarity Index: 0.999588
Library: C:\CLASS-VP\Spec\CFDmp.lib

Degradation study in Photochemical Condition-1

To check the degradation of Cefdinir in ultra violet radiation. The study carried out in solid state under UV light.

Experimental Condition : 5.00gm of Cefdinir was taken into a open petridish and kept under UV radiation. The sample was analysed before exposing to UV radiation, after 12 hrs & after 24 hrs of exposure to UV radiation.

Initial Solution : 0.1014gm of Cefdinir was weighed and diluted to 100ml with dilution buffer. Pipette out 10ml from above solution immediately after preparation & diluted to 50ml with mobile buffer.

Sample Solution after 12 hrs : 0.1015gm of Cefdinir from UV light exposed petridish was weighed and diluted to 100ml with dilution buffer. Pipette out 10ml from above solution & diluted to 50ml with mobile phase.

Sample Solution after 24 hrs : 0.1500gm of Cefdinir from UV light exposed petridish was weighed and diluted to 100ml with water. Pipette out 10ml from above solution & diluted to 50ml with water

Solid, exposed in UV light :-

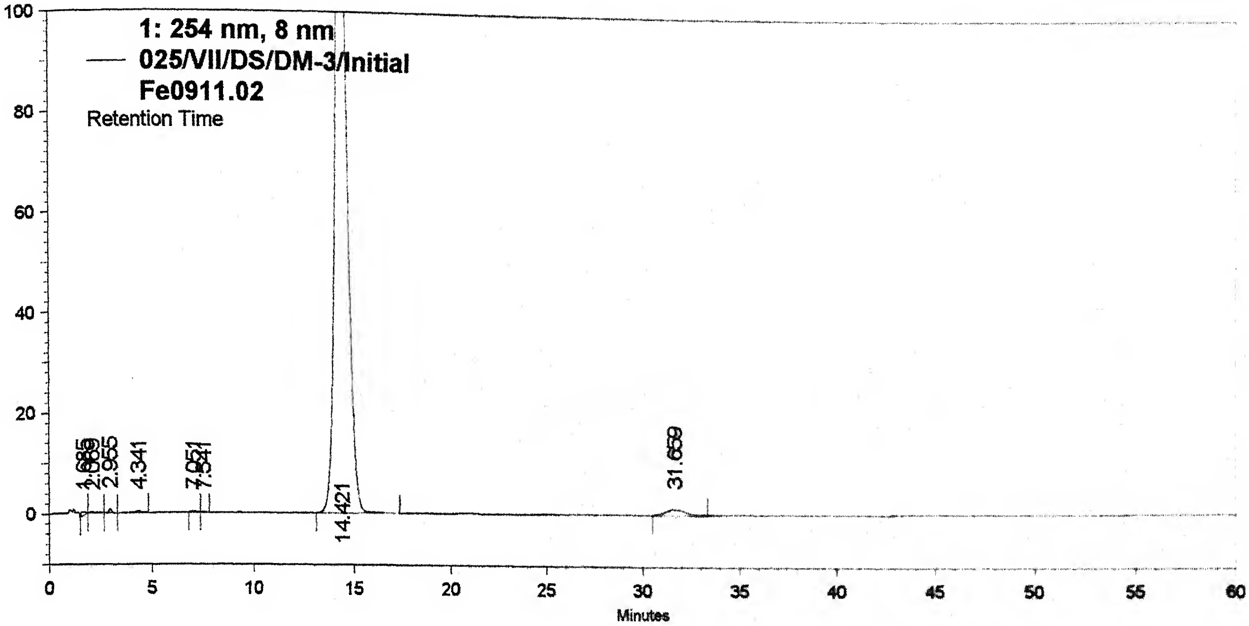
S.No.	Time	Area	% Variance	File Name
01.	Initial	5736764		FE0911.02
02.	After 12Hrs.	5663472	1.28	FE0911.13
03.	After24 Hrs.	5545834	3.33	FE0911.15

Conclusion :

(1) Spectrum correlation of Cefdinir peak at initial & after 24.0hrs showed correlation more than 99%. Which confirms that no other peak is merging with Cefdinir.

(2) Contour plot of Cefdinir peak from initial to final analysis showed same pattern confirming that no other peak is merging with the main peak of Cefdinir.

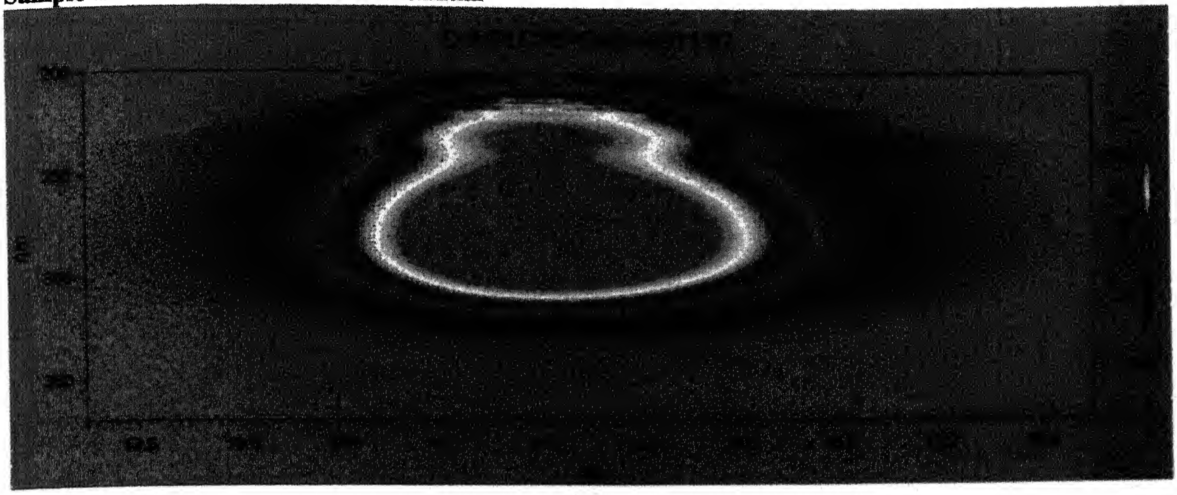
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe0911.02
Acquired Time : 11/9/00 8:32:34 AM
Sample ID : 025/VII/DS/DM-3/Initial



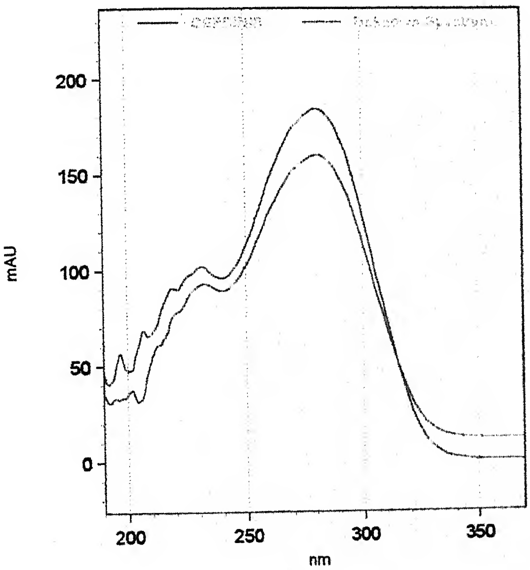
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	1.685	9922	0.17	
2	2.069	4906	0.08	
3	2.955	6641	0.11	
4	4.341	6303	0.11	
5	7.051	2638	0.05	
6	7.541	1153	0.02	
7	14.421	5742742	98.12	CEFDINIR
8	31.659	78268	1.34	
Totals		5852573	100.00	

Method Name : **C:\CLASS-VP\Methods\CEFDVAL.met**
File Name : **D:\HPLC16PDA\Fe0911.02**
Acquired Time : **11/9/00 8:32:34 AM**
Sample ID : **025/VII/DS/DM-3/Initial**

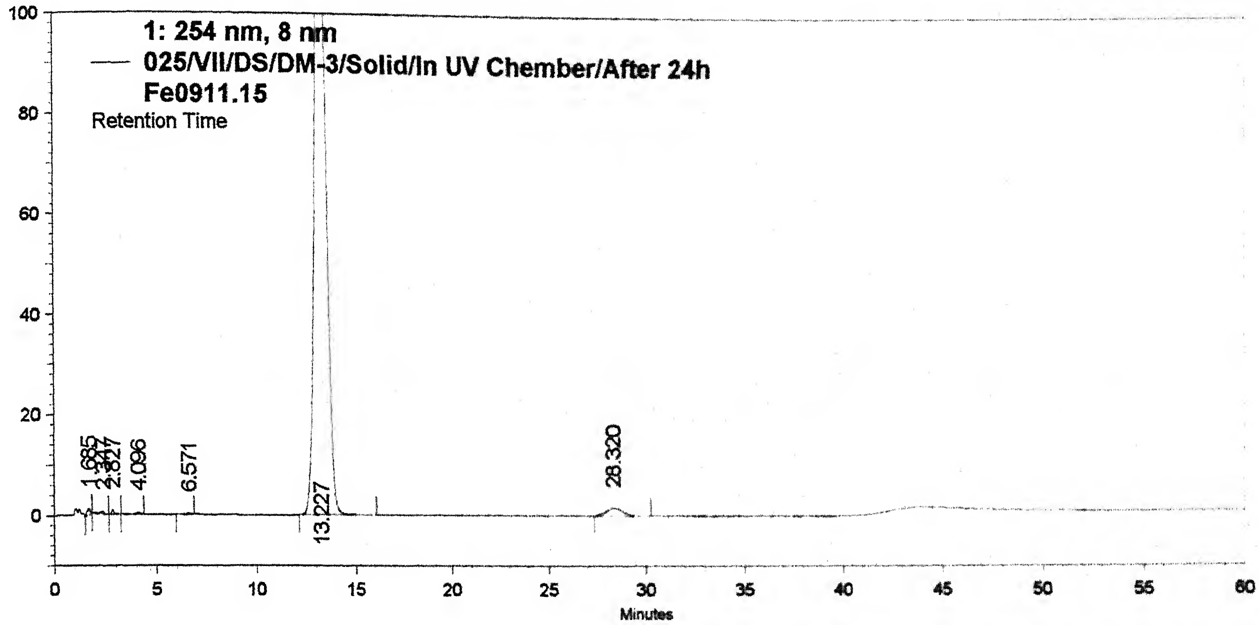


Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.996050
Library: C:\CLASS-VP\Spec\CFDmp.lib

Method Name : **C:\CLASS-VP\Methods\CEFDVAL.met**
File Name : **D:\HPLC16PDA\Fe0911.15**
Acquired Time : **11/10/00 9:56:20 AM**
Sample ID : **025/VII/DS/DM-3/Solid/In UV Chember/After 24h**

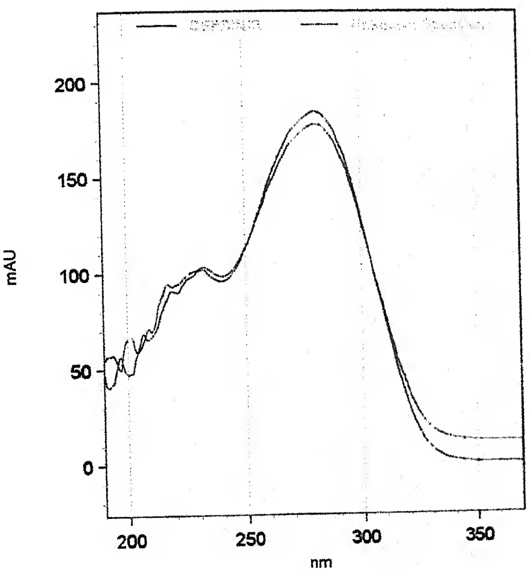


1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	1.685	13356	0.24	
2	2.347	8337	0.15	
3	2.827	7317	0.13	
4	4.096	7363	0.13	
5	6.571	4060	0.07	
6	13.227	5550533	97.73	CEFDINIR
7	28.320	88591	1.56	
Totals		5679557	100.00	

Method Name : **C:\CLASS-VP\Methods\CEFDVAL.met**
File Name : **D:\HPLC16PDA\Fe0911.15**
Acquired Time : **11/10/00 9:56:20 AM**
Sample ID : **025/VII/DS/DM-3/Solid/In UV Chember/After 24h**

Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.999508
Library: C:\CLASS-VP\Spec\CFDmp.lib

Degradation study in Photochemical Condition-2

To check the degradation of Cefdinir in ultra violet radiation. The study carried out in solution under UV light.

Stock Solution :0.1002gm of Cefdinir was weighed and diluted to 100ml with dilution buffer. Expose this solution under UV light for 24Hrs.

Initial Solution :Pipetted 10ml of stock solution & diluted to 50ml with mobile phase.

Solution after 12 hrs :Pipetted 10ml of stock solution & diluted to 50ml with mobile phase.

Solution after 24 hrs :Pipetted 10ml of stock solution & diluted to 50ml with mobile phase.

Solution, exposed in UV light :-

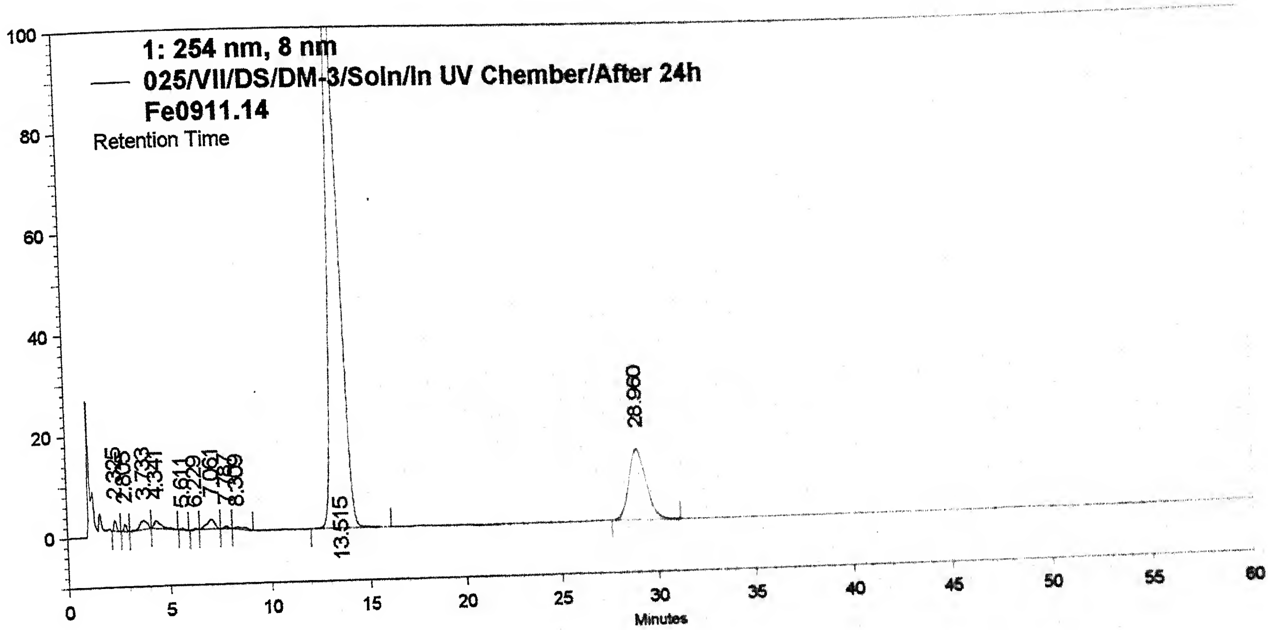
S.No.	Time	Area	% Variance	File Name
01.	Initial	5736764		FE0911.02
02.	After 12Hrs.	5020086	12.49%	FE0911.15
03.	After24 Hrs.	4365081	23.91%	FE0911.14

Conclusion :

(1) Spectrum correlation of Cefdinir peak at initial & after 24.0hrs showed correlation more than 99%. Which confirms that no other peak is merging with Cefdinir

(2) Contour plot of Cefdinir peak from initial to final analysis showed same pattern confirming that no other peak is merging with the main peak of Cefdinir

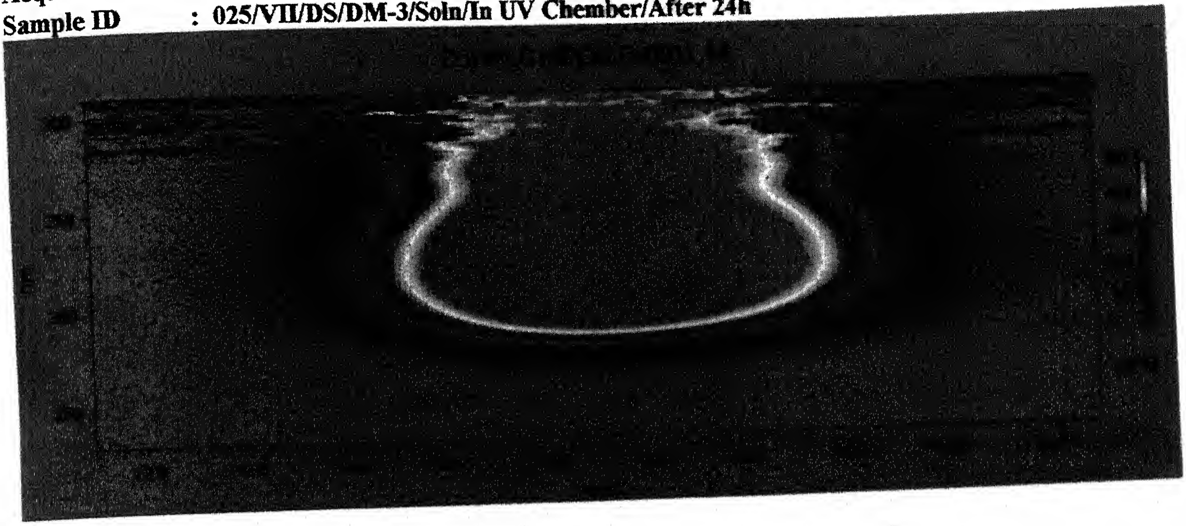
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe0911.14
Acquired Time : 11/10/00 8:52:06 AM
Sample ID : 025/VII/DS/DM-3/Soln/In UV Chember/After 24h



1: 254 nm, 8 nm

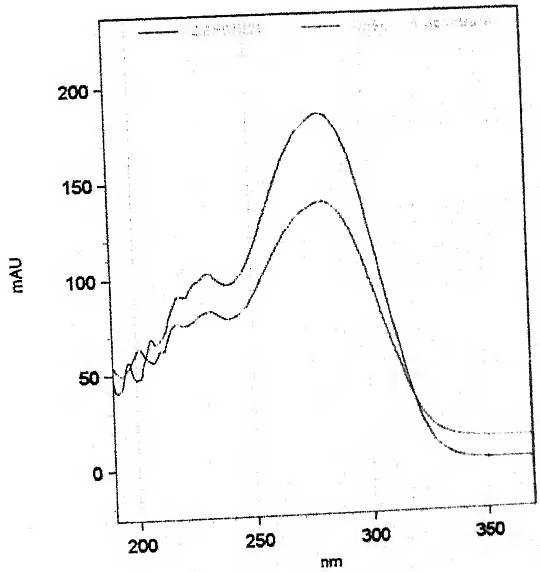
Pk #	Retention Time	Area	Area Percent	Name
1	2.325	19634	0.36	
2	2.805	13129	0.24	
3	3.733	47672	0.88	
4	4.341	40201	0.74	
5	5.611	4730	0.09	
6	6.229	4348	0.08	
7	7.061	52575	0.97	
8	7.787	7265	0.13	
9	8.309	13806	0.25	
10	13.515	4362306	80.12	CEFDINIR
11	28.960	879160	16.15	
Totals		5444826	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe0911.14
Acquired Time : 11/10/00 8:52:06 AM
Sample ID : 025/VII/DS/DM-3/Soln/In UV Chember/After 24h



Number of Hits: 1

Peak Name: CEFDINIR



Hit 1
Name: CEFDINIR
Similarity Index: 0.998732
Library: C:\CLASS-VP\Spec\CFDmp.lib

Degradation study in Acidic Condition

To check the degradation of Cefdinir in Acidic condition. The study carried out in 1N HCl

Solution preparation :

Stock solution : 0.1009gm of Cefdinir was weighed in 100ml volumetric flask. Dissolved in 10ml pH=7 buffer and then make up to the mark with 1N HCl.

Initial solution : 10ml from stock solution was pipetted immediately after preparation & diluted to 50ml with mobile phase.

Subsequent solutions:10ml from stock solution was pipetted & diluted to 50ml after every hour with mobile phase up to 3hrs.

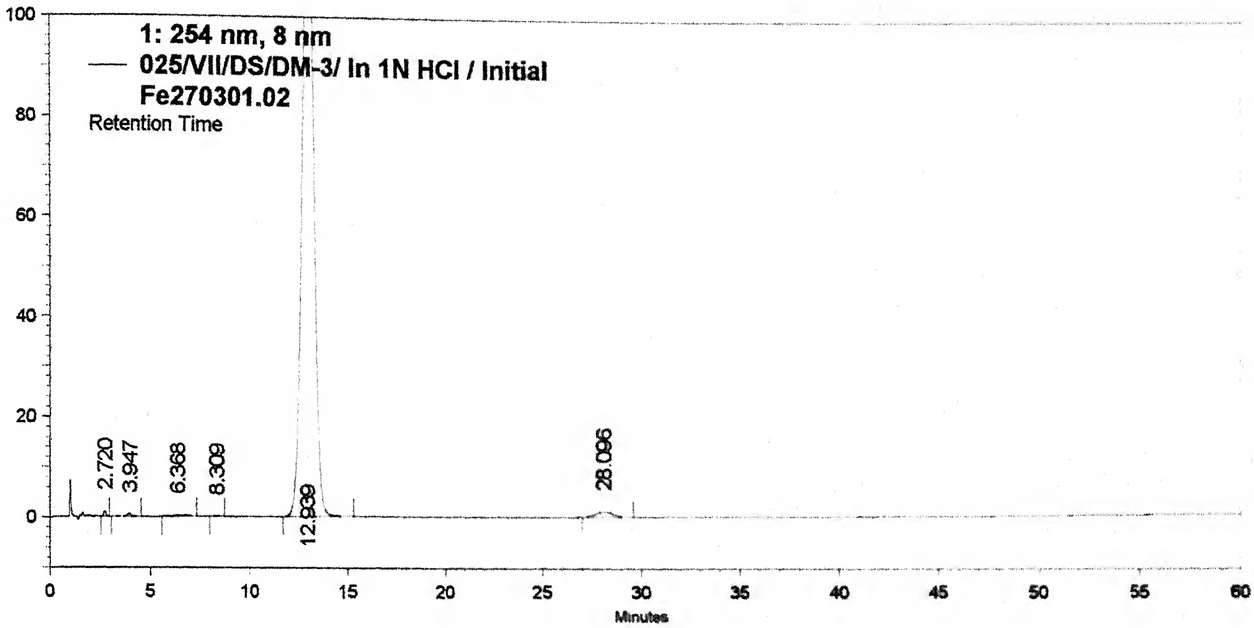
S.No.	Time	Area	% Variance	File Name
01.	Initial	5291635		FE270301.02
02.	After 1hr	5256288	0.67	FE270301.03
03.	After 2 hr	5242356	0.93	FE270301.04
04.	After 3 hr	5194644	1.83	FE270301.05

Conclusion :

(1) Spectrum correlation of Cefdinir peak at initial & after 3.0hrs shows correlation more than 99%. Which confirms that no other peak is merging with Cefdinir.

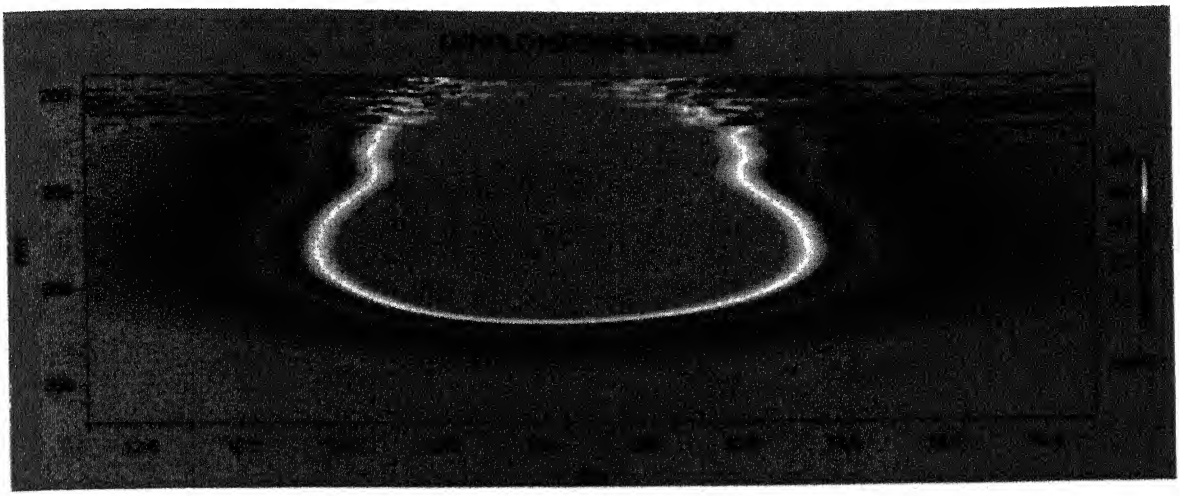
(2) Contour plot of Cefdinir peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefdinir.

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe270301.02
Acquired Time : 3/27/01 1:10:48 PM
Sample ID : 025/VII/DS/DM-3/ In 1N HCl / Initial

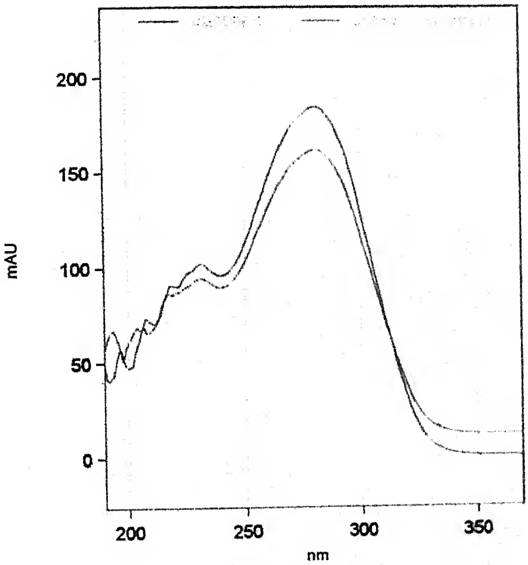


1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	2.720	9110	0.17	
2	3.947	10376	0.19	
3	6.368	12882	0.24	
4	8.309	3359	0.06	
5	12.939	5276758	98.05	CEFDINIR
6	28.096	69065	1.28	
Totals		5381550	100.00	

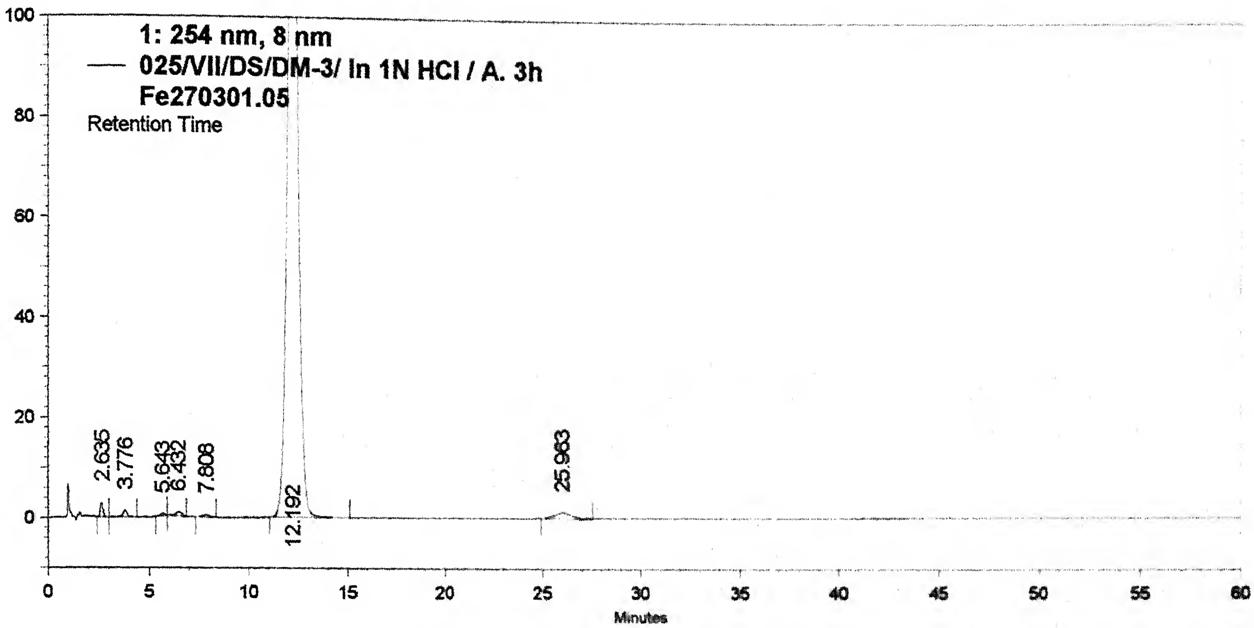


Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.998943
Library: C:\CLASS-VP\Spec\CFDmp.lib

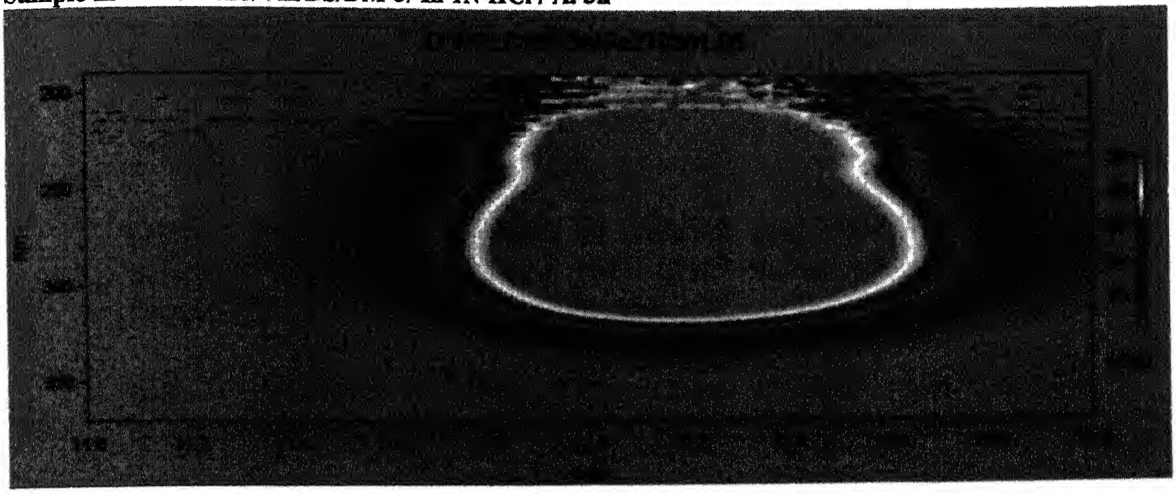
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe270301.05
Acquired Time : 3/27/01 4:32:22 PM
Sample ID : 025/VII/DS/DM-3/ In 1N HCl / A. 3h



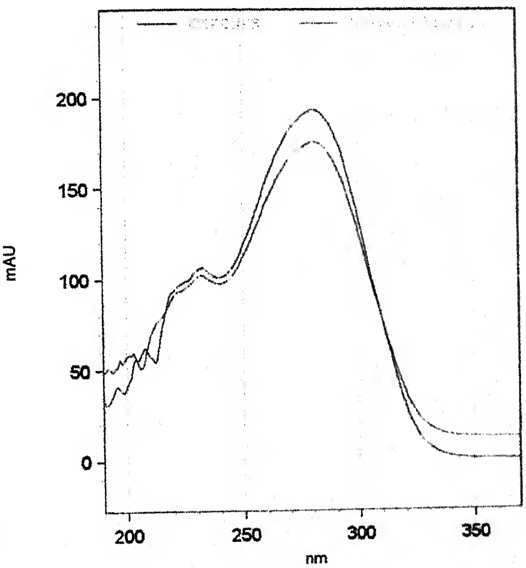
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	2.635	23767	0.44	
2	3.776	19682	0.37	
3	5.643	7823	0.15	
4	6.432	19460	0.36	
5	7.808	10872	0.20	
6	12.192	5201480	97.20	CEFDINIR
7	25.963	68019	1.27	
Totals		5351103	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe270301.05
Acquired Time : 3/27/01 4:32:22 PM
Sample ID : 025/VII/DS/DM-3/ In 1N HCl / A. 3h



Peak Name: CEFDINIR **Number of Hits: 1**



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Similarity Index: 0.999150
Library: C:\CLASS-VP\Spec\CFDHCl.lib

Degradation study in Alkaline Condition

To check the degradation of Cefdinir in Basic condition. The study carried out in 1N NaOH

Solution preparation :

Stock solution : 0.1024gm of Cefdinir was weighed in 100ml volumetric flask. Dissolved in 10ml pH=7 buffer and 5ml NaOH & make up to the mark with mobile phase.

Initial solution : 10ml from stock solution was pipetted immediately after preparation & diluted to 50ml with mobile phase.

Subsequent solutions:10ml from stock solution was pipetted & diluted to 50ml after every hour with mobile phase up to 3hrs.

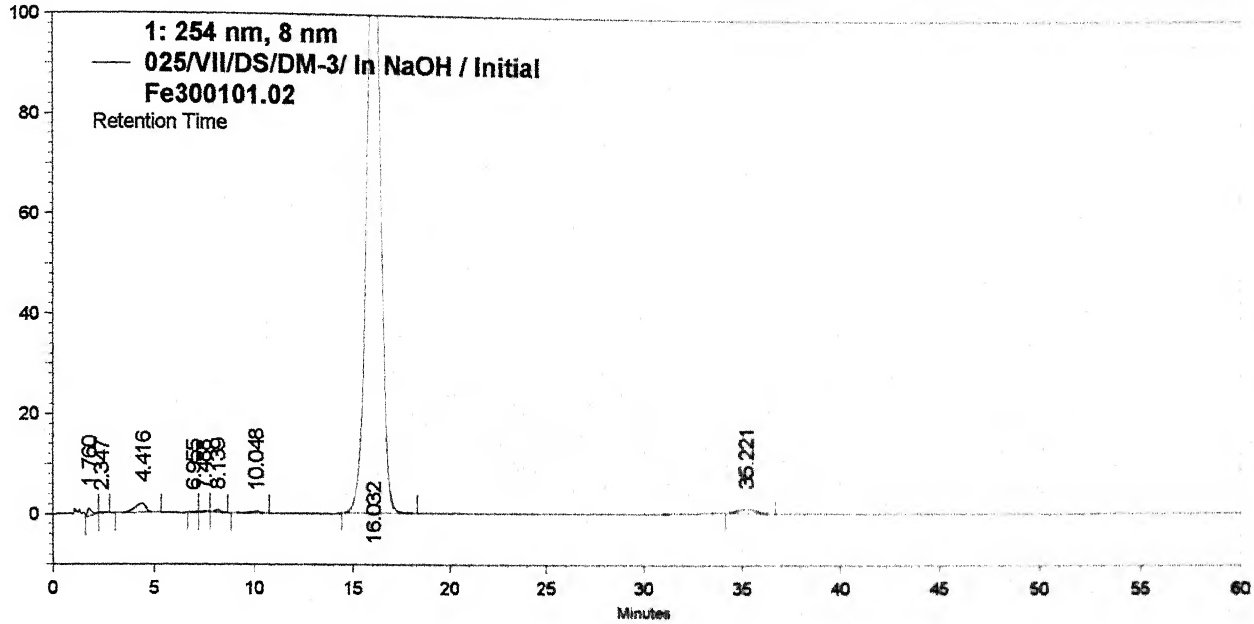
S.No.	Time	Area	% Variance	File Name
01.	Initial	5949924		FE300101.02
02.	After 1hr	3336328	43.92	FE300101.03
03.	After 2 hr	1872346	68.5	FE300101.04
04.	After 3 hr	1582253	73.41	FE300101.05

Conclusion :

(1) Spectrum correlation of Cefdinir peak at initial & after 3.0hrs shows correlation more than 99%. Which confirms that no other peak is merging with Cefdinir.

(2) Contour plot of Cefdinir peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefdinir.

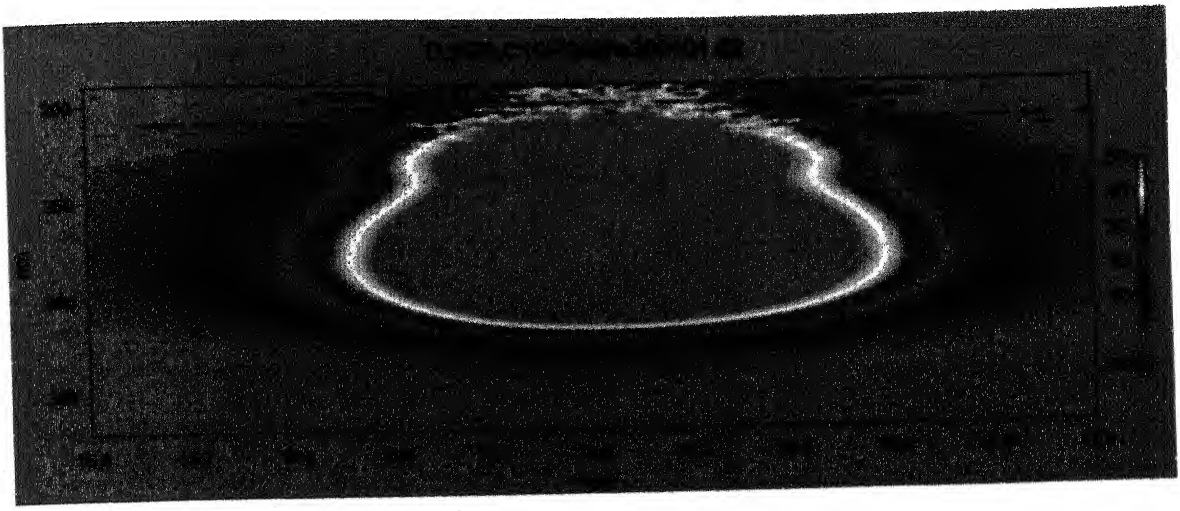
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe300101.02
Acquired Time : 1/30/01 3:05:51 PM
Sample ID : 025/VII/DS/DM-3/ In NaOH / Initial



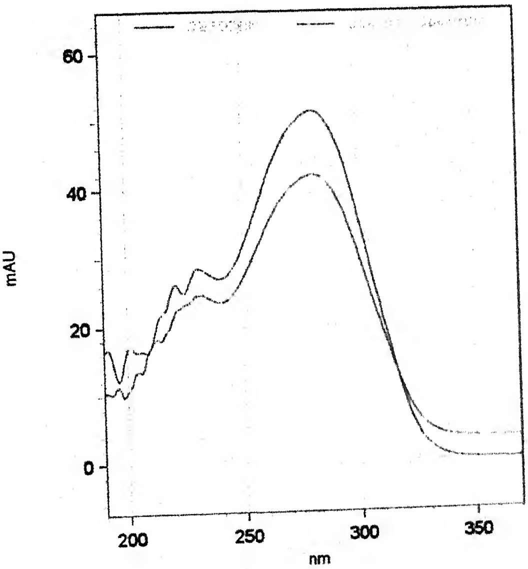
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	1.760	21670	0.35	
2	2.347	3482	0.06	
3	4.416	76720	1.25	
4	6.955	2925	0.05	
5	7.488	2724	0.04	
6	8.139	9770	0.16	
7	10.048	14614	0.24	
8	16.032	5952083	96.88	CEFDINIR
9	35.221	59841	0.97	
Totals		6143829	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe300101.02
Acquired Time : 1/30/01 3:05:51 PM
Sample ID : 025/VII/DS/DM-3/ In NaOH / Initial

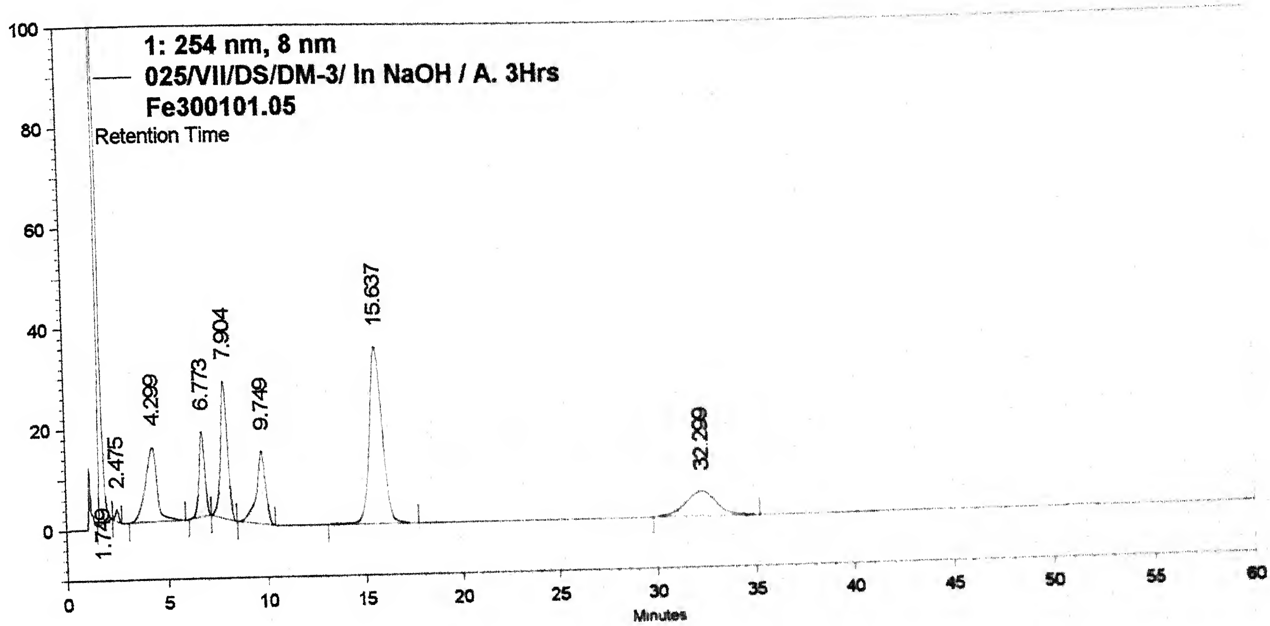


Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.998328
Library: C:\CLASS-VP\Spec\CFDbasic.lib

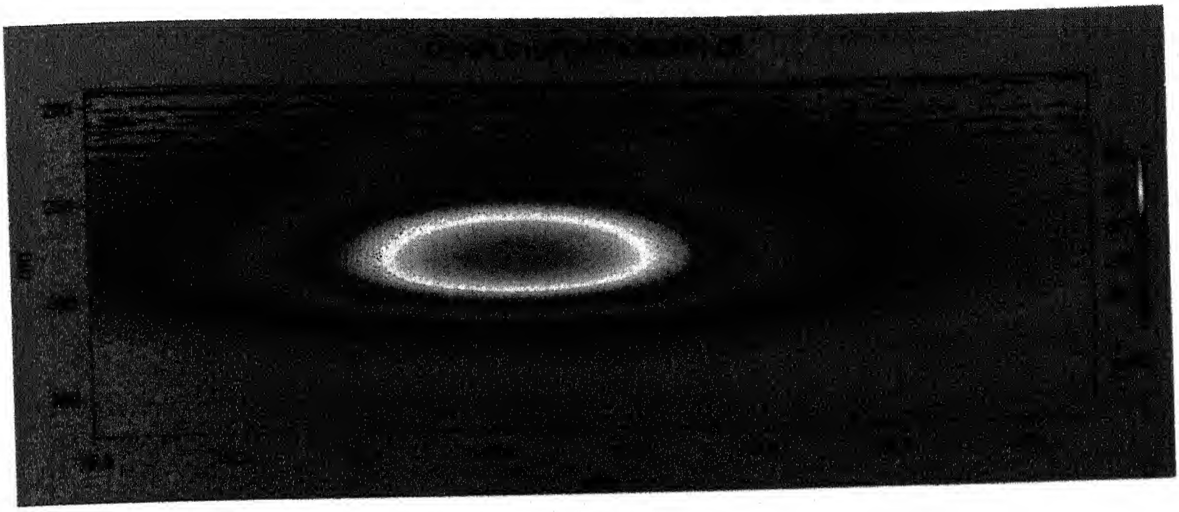
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe300101.05
Acquired Time : 1/30/01 6:16:32 PM
Sample ID : 025/VII/DS/DM-3/ In NaOH / A. 3Hrs



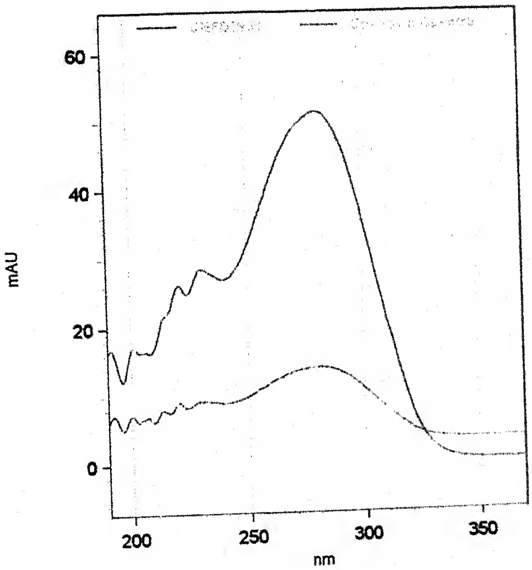
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	1.749	1157622	21.28	
2	2.475	26098	0.48	
3	4.299	618842	11.38	
4	6.773	351972	6.47	
5	7.904	652276	11.99	
6	9.749	484351	8.90	
7	15.637	1602608	29.46	CEFDINIR
8	32.299	545348	10.03	
Totals		5439117	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe300101.05
Acquired Time : 1/30/01 6:16:32 PM
Sample ID : 025/VII/DS/DM-3/ In NaOH / A. 3Hrs



Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.999664
Library: C:\CLASS-VP\Spec\CFDbasic.lib

Degradation study in Oxidative Condition

To check the degradation of Cefdinir in Oxidative condition. The study carried out in Hydrogen peroxide (20%).

Solution preparation :

Stock solution : 0.1016gm of Cefdinir was weighed in 100ml volumetric flask dissolved it in dilution buffer in 0.5ml Hydrogen peroxide (30%) then make up to the mark with dilution buffer.

Initial solution : 10ml from stock solution was pipetted immediately after preparation & diluted to 50ml with mobile phase.

Subsequent solutions: 10ml from stock solution was pipetted & diluted to 50ml after every hour with mobile phase up to 3hrs.

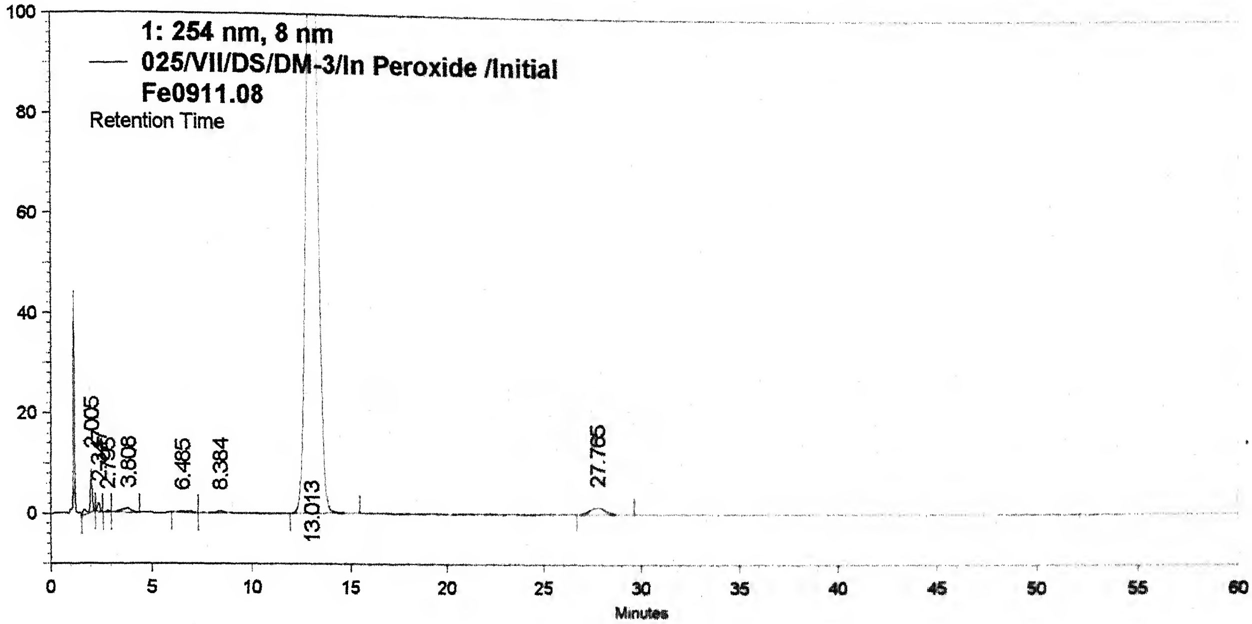
S.No.	Time	Area	% Variance	File Name
01.	Initial	5645424		FE0911.08
02.	After 1hr	4310124	23.65	FE0911.09
03.	After 2 hr	3191902	43.46	FE0911.10
04.	After 3 hr	2330581	58.72	FE0911.11

Conclusion :

(1) Spectrum correlation of Cefdinir peak at initial & after 3.0hrs shows correlation more than 99%. Which confirms that no other peak is merging with Cefdinir.

(2) Contour plot of Cefdinir peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefdinir.

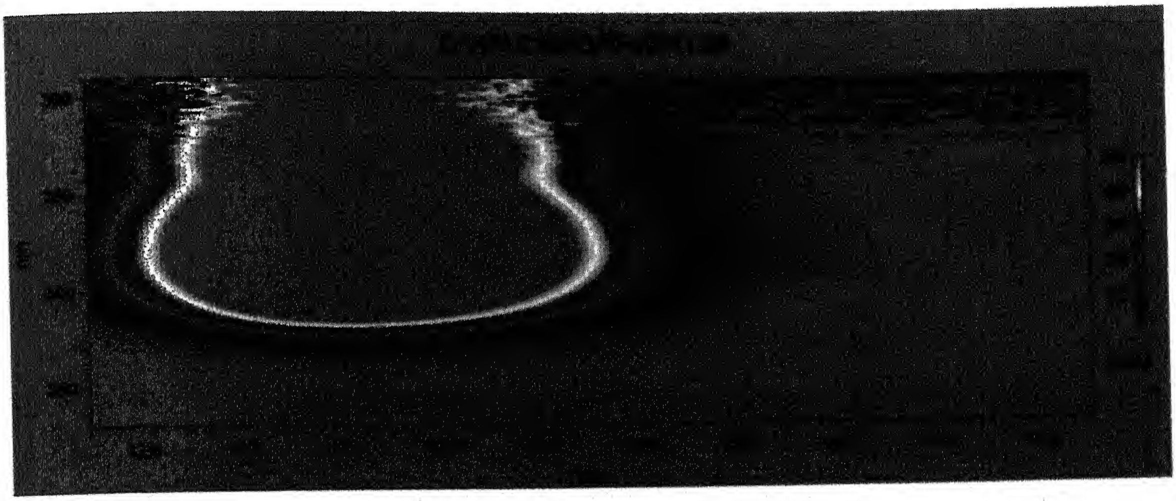
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe0911.08
Acquired Time : 11/9/00 3:10:01 PM
Sample ID : 025/VII/DS/DM-3/In Peroxide /Initial



1: 254 nm, 8 nm

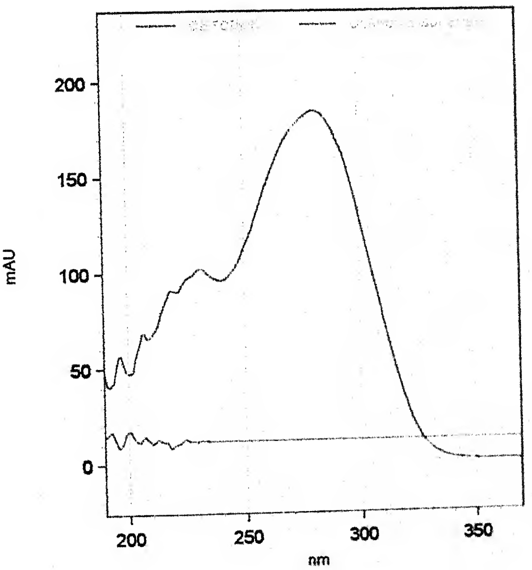
Pk #	Retention Time	Area	Area Percent	Name
1	2.005	72399	1.24	
2	2.347	14013	0.24	
3	2.795	4101	0.07	
4	3.808	27730	0.47	
5	6.485	12348	0.21	
6	8.384	10996	0.19	
7	13.013	5639025	96.25	CEFDINIR
8	27.765	78316	1.34	
Totals		5858928	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe0911.08
Acquired Time : 11/9/00 3:10:01 PM
Sample ID : 025/VII/DS/DM-3/In Peroxide /Initial



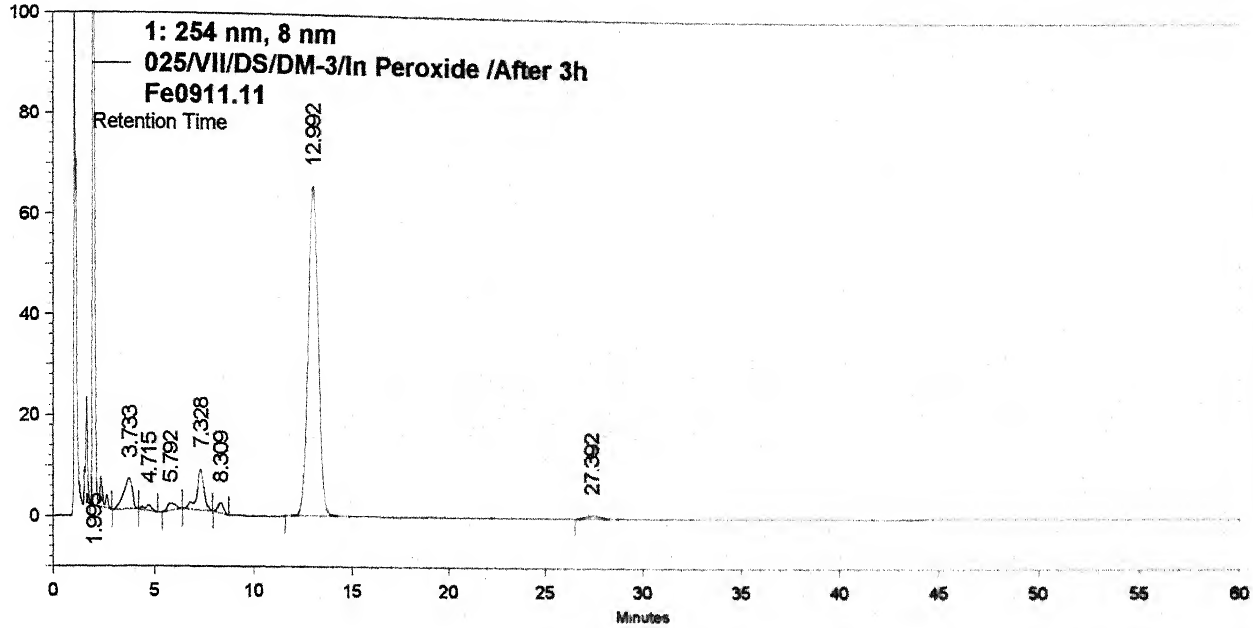
Peak Name: CEFDINIR

Number of Hits: 1



Hit 1
Name: CEFDINIR
Similarity Index: 0.138843
Library: C:\CLASS-VP\Spec\CFDmp.lib

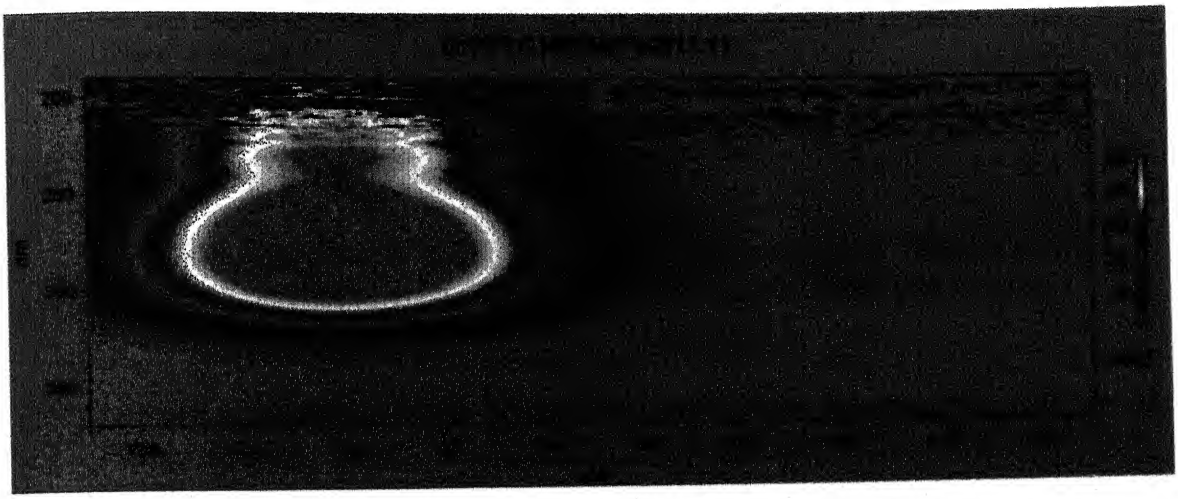
Method Name : **C:\CLASS-VP\Methods\CEFDVAL.met**
File Name : **D:\HPLC16PDA\Fe0911.11**
Acquired Time : **11/9/00 6:34:13 PM**
Sample ID : **025/VII/DS/DM-3/In Peroxide /After 3h**



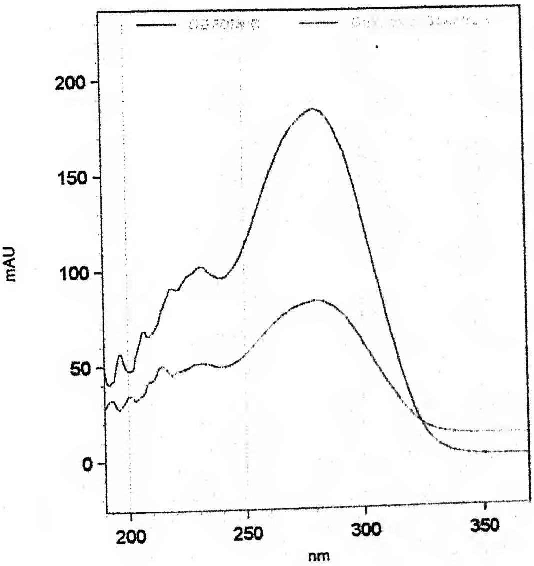
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	1.995	1214293	29.78	
2	3.733	184719	4.53	
3	4.715	27622	0.68	
4	5.792	42925	1.05	
5	7.328	207820	5.10	
6	8.309	40725	1.00	
7	12.992	2324735	57.02	CEFDINIR
8	27.392	34498	0.85	
Totals		4077337	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe0911.11
Acquired Time : 11/9/00 6:34:13 PM
Sample ID : 025/VII/DS/DM-3/In Peroxide /After 3h



Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.999028
Library: C:\CLASS-VP\Spec\CFDmp.lib

Degradation study on Thermal Condition

To check the degradation of Cefdinir after heating at about 50°C +/-5°C for 3 hours.

Solution preparation :

Stock solution :0.1008gm of Cefdinir was weighed. Dissolved and diluted to 100ml with dilution buffer. Keep the stock solution in water bath at 50°C for 3.0 hrs.

Initial solution : 10ml from stock solution was pipetted out immediately after preparation & diluted to 50ml with mobile phase.

Subsequent solutions:10ml from stock solution was pipetted & diluted to 50ml after every hour with mobile phase.

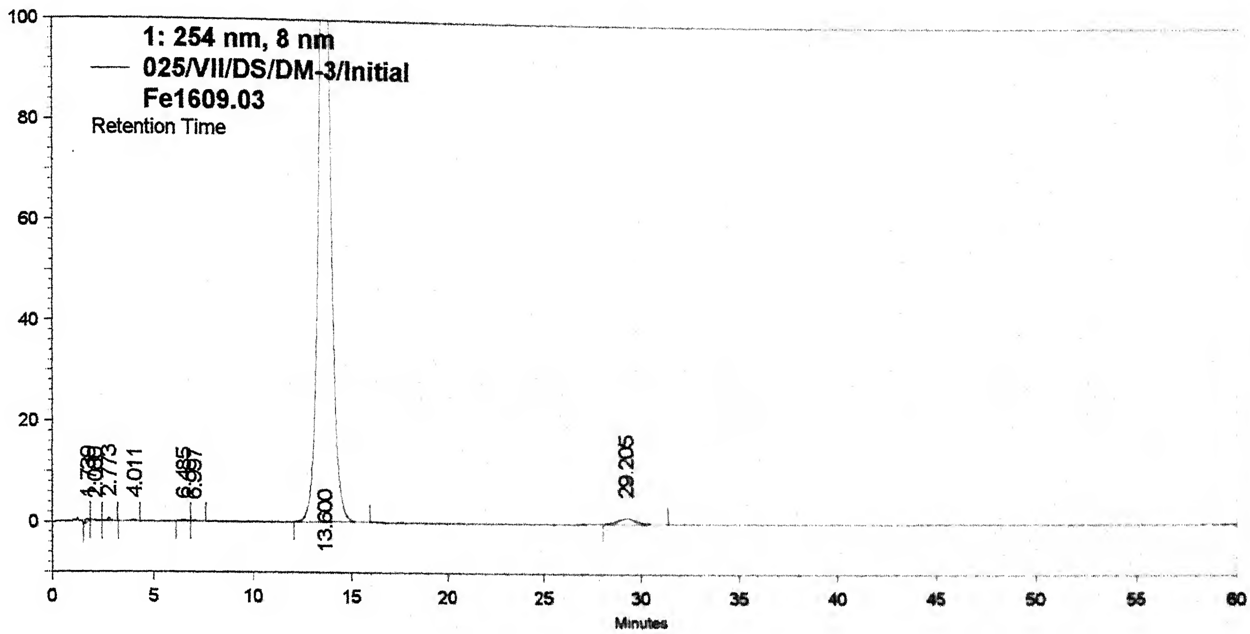
S.No.	Time	Area	% Variance	File Name
01.	Initial	5801115		FE1609.03
02.	After 1Hr.	5738572	1.08	FE1609.04
03.	After2 Hrs.	5719598	1.40	FE1609.05
04.	After3 Hrs.	5694473	1.84	FE1609.06

Conclusion :

(1) Spectrum correlation of Cefdinir peak at initial & after 3.0hrs shows correlation more than 99%. Which confirms that no other peak is merging with Cefdinir.

(2) Contour plot of Cefdinir peak from initial to final analysis shows same pattern confirming that no other peak is merging with the main peak of Cefdinir.

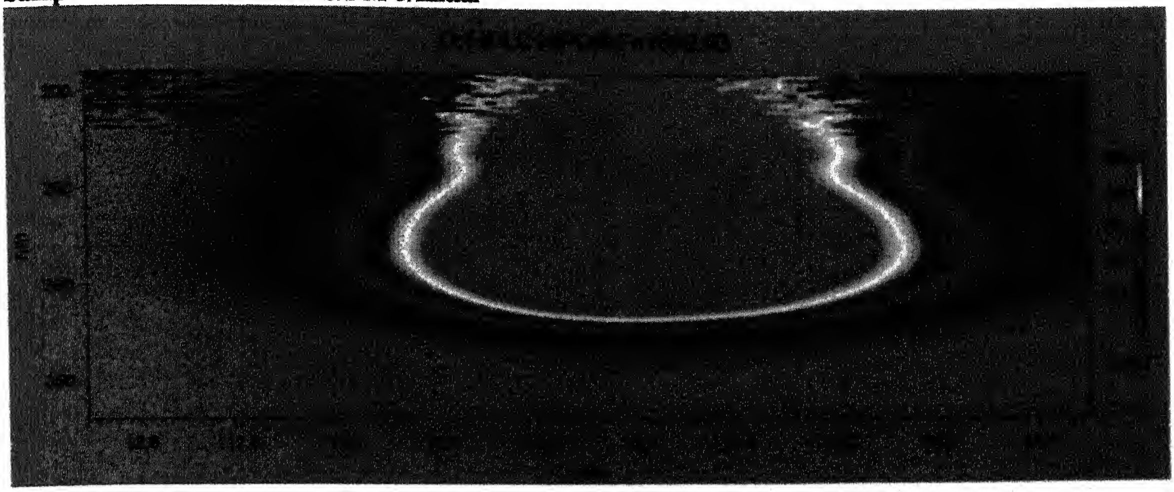
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1609.03
Acquired Time : 9/16/00 10:59:11 AM
Sample ID : 025/VII/DS/DM-3/Initial



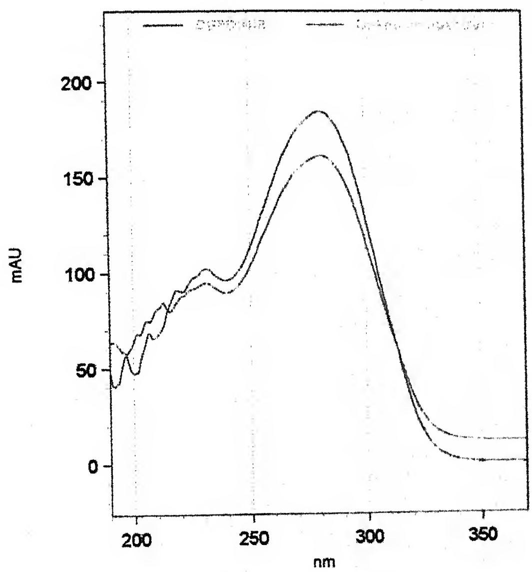
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	1.739	8482	0.14	
2	2.080	2134	0.04	
3	2.773	6002	0.10	
4	4.011	5390	0.09	
5	6.485	5180	0.09	
6	6.997	1377	0.02	
7	13.600	5792871	98.23	CEFDINIR
8	29.205	75988	1.29	
Totals		5897424	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1609.03
Acquired Time : 9/16/00 10:59:11 AM
Sample ID : 025/VII/DS/DM-3/Initial

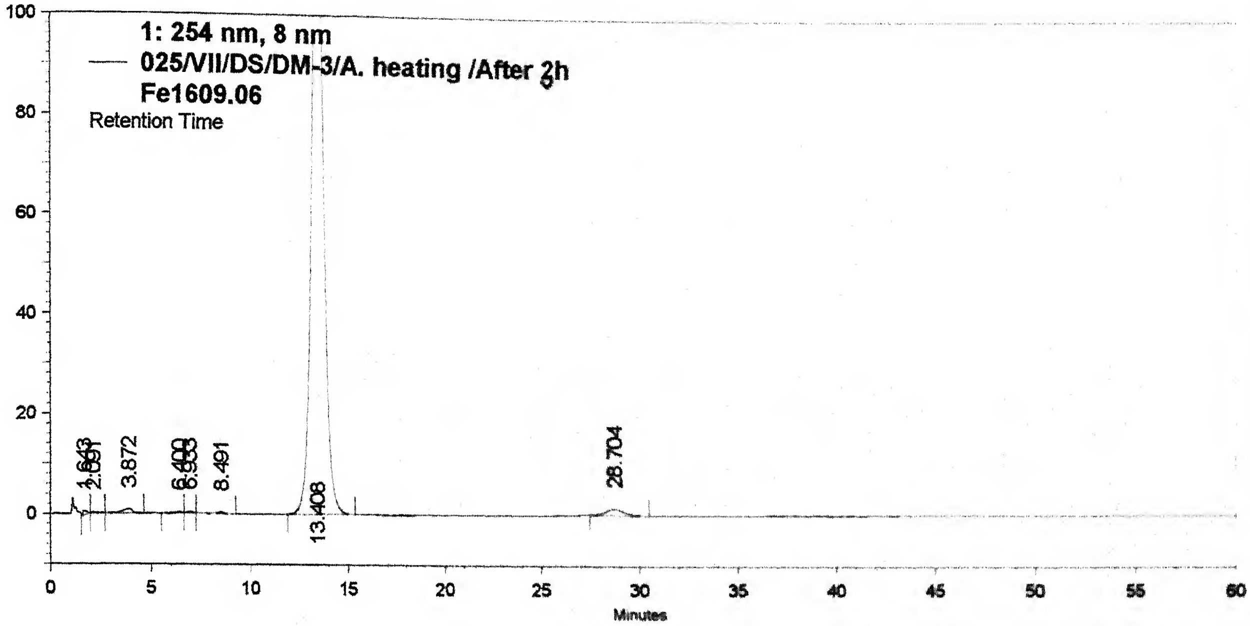


Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.998412
Library: C:\CLASS-VP\Spec\CFDmp.lib

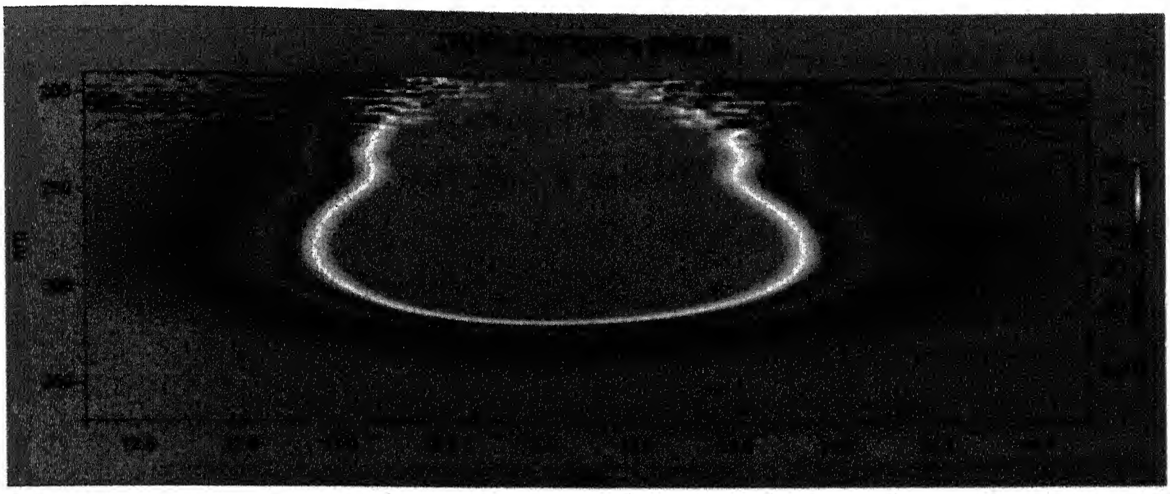
Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1609.06
Acquired Time : 9/16/00 2:22:43 PM
Sample ID : 025/VII/DS/DM-3/A. heating /After 3h



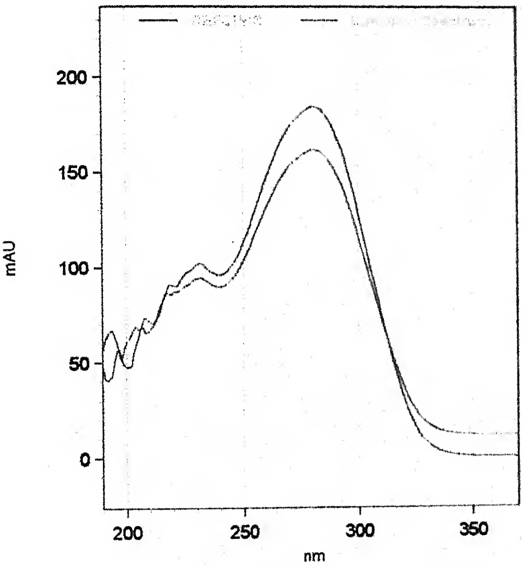
1: 254 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name
1	1.643	11724	0.20	
2	2.091	3773	0.06	
3	3.872	29101	0.50	
4	6.400	6479	0.11	
5	6.933	4969	0.09	
6	8.491	9265	0.16	
7	13.408	5681540	97.56	CEFDINIR
8	28.704	76966	1.32	
Totals		5823817	100.00	

Method Name : C:\CLASS-VP\Methods\CEFDVAL.met
File Name : D:\HPLC16PDA\Fe1609.06
Acquired Time : 9/16/00 2:22:43 PM
Sample I : 025/VII/DS/DM-3/A. heating / after 3h



Peak Name: CEFDINIR **Number of Hits: 1**



Hit 1
Name: CEFDINIR
Similarity Index: 0.998943
Library: C:\CLASS-VP\Spec\CFDmp.lib

IMPURITY PROFILING OF CEFDINIR

Impurity profile (Related substance test) is the most important test for any compound because now days it is mandatory that any impurity, which is in amount 0.1% or more, should be isolated and characterized. This test is carried out by liquid chromatography method, which provides valuable information about quality of product and the level of impurities before and after stability study.

Cefdinir is a new orally effective semisynthetic cephalosporin and its higher antimicrobial activity against gram negative and positive bacteria is due to vinyl group at C-3 and a (Z)-2-(amino-4-thiazolyl)-2-(hydroxyimino)acetyl moiety at C-7 position.

In cefdinir total 10 impurities were isolated and identified. These impurities were formed by forced degradation on heating the sample at 100°C for 72 hrs, and isomerization in acidic pH (1.0), neutral pH (6.0), and basic pH (12.0).

EXPERIMENTAL SECTION

Materials

Cefdinir Batch No#08006 of Lupin ltd was used and all other chemicals used were of reagent grade. Water was purified by milli-Q System.

Procedures

The following instruments were used for the impurity profiling of cefdinir:

Instruments : IR spectra were recorded on Perkin-Elmer 1650(USA) and Shimadzu FT-IR 8201(Japan) model, NMR spectra were recorded on Bruker DRX-200 MHz(Switzerland) and Mass spectra were recorded on PE –SCIEX API-3000(USA) triple quadruple. The pH of solution was measured using Control Dynamic(India). The balance was Mettler –AE-250(Switzerland)

Analytical System : Shimadzu binary gradient HPLC equipped with

- System controller – SCL 10 ATvp
- Two pumps - LC – 10ATvp
- Degasser unit - DGU – 14A
- UV/VIS. Detector – SPD – 10 Avp
- Autosampler - SIL – 10 ADvp fitted with 50 μl sample loop
- Column oven - 10 Asvp
- Data processor - Class-VP –5.03 software

To monitor all impurities fractions of MPLC and preparative HPLC by analytical HPLC, following condition was used:

Column	:TSK Gel ODS 80(75*4.6mm,5.0μm)
Wavelength	: 254nm
Mobile phase	: Buffer:(10mM TBAH + 0.04mM. EDTA) + Methanol
Composition	: 17:3
PH	: 7.0 by H_3PO_4
Flow rate	:1.50 ml/min

Preparative HPLC

- Two pumps - LC- 8A
- One UV/Vis. detector - SPD 10-A
- Column -G.L.Science-Inertsil-C-18(250mm*20mm,8.0μ)

Medium Pressure Liquid Chromatography :

- System controller Labomat-VS-200
- Pump - Laboprep MD-50
- UV detector - UVIS-201
- Column - 700mm * 50mm Glass (Packed by C-18, 20-40 μm particle.)

Lyophilizer: Virtis EL2500

EXPERIMENTAL WORK

Isolation of cefdinir impurity-A

Charged cefdinir (06 gm) in round bottom flask Followed by water (150 ml) and pH was adjusted to 9.8 with ammonia solution, resulted solution was kept at 27-28°C for 19 hrs. pH was adjusted to 3.0 with 2M HCl and kept at 27-28°C for 30mins, this solution was lyophilized and loaded on MPLC.

The mobile phase used was as follows:

Buffer : 0.1 M Ammonium acetate

Solution A : Buffer + Acetonitrile in ratio of (90:10)

Solution B : Buffer + Acetonitrile in ratio of (50:50)

pH : As such

Wavelength : 254nm

Flow rate : 100-ml/ min

Column : 700mm x 50mm diameter with C18 silica of 20-40μm

MPLC was used for initial enrichment of the impurity sample up to about 25% (by HPLC area method) employing following gradient program.

Time	Event	Value	Flow ml/min
00.01	B.conc	00.0	50.00
30.00	B.conc	00.0	50.00
50.00	B.conc	100.0	100.00
80.00	B.conc	00.0	100.00
100.00	B.conc	00.0	100.00
101.00	B.conc	00.0	Stop

All fractions collected were monitored using the above analytical method. The fractions containing impurity were concentrated using rotavapour at bath temperature 38-40°C in high vacuum to distill out the acetonitrile. The resulted solution was lyophilized, and there after loaded on preparative HPLC for further purification.

Purification of lyophilized fractions of MPLC by preparative HPLC.

The mobile phase used was as follows:

Mobile phase:		Aqueous acetic acid 0.3% + Acetonitrile in ratio of (90:10)
pH	:	As such
Wavelength	:	254nm
Flow rate	:	15 ml/min
Column	:	Inertsil ODS 3-V 250mm x 20mm and 08μm particle size

The fractions containing the impurity were collected and lyophilized. The solid mass was taken for further evaluation of the purity and the spectroscopic studies.

The PMR spectrum was recorded in DMSO d_6. The mass spectrum was recorded by dissolving the sample in acetonitrile/water using atmospheric pressure ionization having turbolon ionization source. The declustrring potential was set at 10, focusing potential at 80, and ionization volt energy (IVE) 4500 at 400°C were set.

Conclusion

The identical observation found in 1H-NMR & MS for both the impurities therefore the structure of impurities A and A1 was assigned as distereoisomers of cefdinir formed due to lactonization between 2-carboxyl and 3-vinyl group.

PMR Spectra

The PMR spectra in DMSO d6 confirm the structure of above-mentioned impurity

S.No.	**Chemical shift (ppm)**	**Multiplicity**	**Assignment**
1.	1.42	d	$-CH_3$
2.	3.78	s	SCH_2
3.	5.15	d	6-H
4.	5.29	q	Lactone CH
5.	5.93	dd	7-H
6.	6.66	s	H of Ar
7.	7.11	br. s	NH_2
8.	9.50	d	CONH
9.	11.33	br. s	NOH

The above spectral data shows vinyl proton signals of cefdinir disappeared and two signals assigned to the $–CHCH_3$ moiety were found suggesting that it contain γ-lactone derived from lactonization between 2-carboxyl and 3-vinyl group.

Mass Spectra

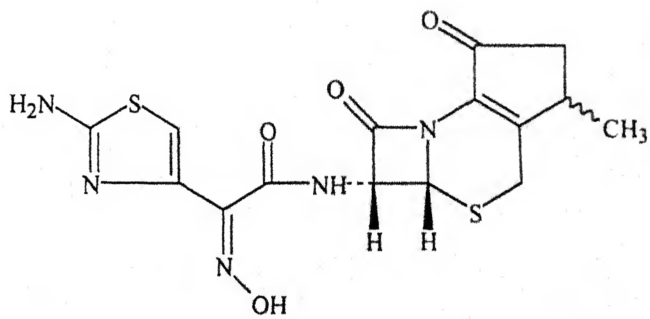
The assignment to the molecular ion peak given below:

S.No.	**m/z amu**	**Structural**
1	396 (M+H)+	Molecular ion

The mass spectra show same mass number as that of cefdinir, confirm the isomerization of cefdinir.

Summary

The unidentified impurities A and A1 were isolated from Cefdinir using MPLC and preparative HPLC. The structures of these impurities were assigned on the basis of PMR amd Mass spectra. They were characterized as isomeric impurity of (Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino)-N-[(3RS,5aR,6R)-1,4,5a,6-tetrahydro-3-methyl-1,7-dioxo-3H,7H-azeto[2,1-b]furo-[3,4-d][1,3]thiazin-6-yl]acetamide



Isolation of cefdinir impurity C

Charged cefdinir (10 gm) in round bottom flask Followed by water (100 ml) and pH was adjusted to 12.7 with 2M NaOH, resulted solution was kept at 50°C for 30 mins. After cooling pH was adjusted to 1.0 with 2M HCl and kept at 27-28°C for 30 mins, solution was neutralized (pH-7) with 2M NaOH and 20 ml of this solution was loaded on MPLC.

The mobile phase used was as follows:

Buffer : Solution A: Water + acetic acid 1%

Solution B: Buffer: Acetonitrile in ratio of (85:15)

pH : 2.90 As such

Wavelength: 254nm

Flow rate : 50 ml/min for 20 min than 100 ml/ min

Column : 700mm x 50mm diameter packed with C18 silica (20-40μm).

MPLC was used for initial enrichment of the impurity up to about 25% (by HPLC area method). The following gradient program was used:

Time	Event	Value	Flow ml/min
00.01	B.conc	00.0	50.00
20.00	B.conc	00.0	50.00
22.00	B.conc	100.0	100.00
52.00	B.conc	100.0	100.00
55.00	B.conc	00.0	100.00
75.00	B.conc	00.0	100.00
76.00	B.conc	00.0	Stop

All fractions collected were monitored using the above analytical method. The fractions containing impurity were concentrated using rotavapour at bath temperature 38-40°C using high vacuum to distill the acetonitrile. The solution was lyophilized, and there after loaded on preparative HPLC for further purification.

Purification of lyophilized fractions of MPLC by preparative HPLC

The mobile phase used was as follows:

Solution A : Aqueous acetic acid 1% + Acetonitrile (85:15)

Solution B : Aqueous acetic acid 0.1% + Acetonitrile (50:50)

pH : 2.90 As such

Wavelength : 254nm

Flow rate : 15 ml/min

Column : Inertsil ODS 3-V 250mm x 20mm (8µm particle size)

The following gradient program was used:

Time	Event	Value	Flow ml/min
00.01	B.conc	00.0	15.0
05.00	B.conc	00.0	15.0
30.00	B.conc	100.0	15.0
40.00	B.conc	100.0	15.0
45.00	B.conc	00.0	15.0
50.00	B.conc	00.0	15.0
51.00	B.conc	Stop	15.0

The fractions containing the impurity were collected and lyophilized. The solid mass was taken for further evaluation of the purity and the spectroscopic studies.

The PMR spectrum was recorded in DMSO d_6. The mass spectrum was recorded by dissolving sample in acetonitrile/water using atmospheric pressure ionization having turbolon ionization source. The declustrring potential was set at 10, focusing potential at 80, and ionization volt energy (IVE) 4500 at 400°C was used.

Conclusion

The structure of impurities C,C1,C2,and C3 were found as isomeric impurity of cefdinir formed due to cyclization taken place between 2-carboxyl and 3-vinyl group and the opening of beta lactam ring. The

distereoisomerism was explained on basis of the isomerization of the γ-lactone methyl.

PMR Spectra

The PMR spectra in DMSO d6 confirm the structure of above-mentioned impurity

S.No.	hemical shift (ppm)	Multiplicity	Assignment
1)	1.35	d	$-CH_3$
2)	3.63	s	SCH_2
3)	4.77	dd	6-H
4)	4.88	dd	7-H
5)	5.11	q	Lactone CH
6)	5.86	d	NH
7)	6.77	s	H of Ar
8)	7.07	br. s	NH_2
9)	8.87	d	CONH
10)	11.21	br. s	NOH

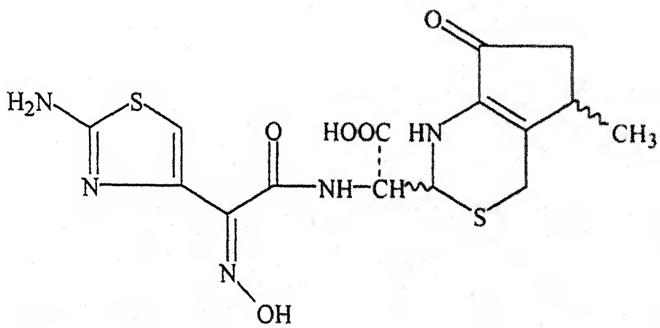
Mass Spectra

The assignment to the molecular ion peak is given below:

S.No.	m/z amu	Structural
1	414 (M+H)+	Molecular ion

Summary

The unidentified impurities C,C1,C2,and C3 were isolated from Cefdinir using MPLC and preparative HPLC. The structure of these impurities were assigned on the basis of PMR and Mass spectra. They were characterized as distereoisomers of (R)-2-[(Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino)acetamido]-2-[(2RS,5RS)-1,2,5,7-tetrahydro-5-methyl-7-oxo-4H-furo[3,4-d]-[1,3]thiazin-2-yl]acetic Acid.



Isolation of cefdinir impurity B

Charged cefdinir (10 gm) in round bottom flask Followed by water (150 ml) and pH was adjusted to 12.7 with 2M NaOH, resulted solution was kept at 50°C for 30min thereafter solution was cooled to ambient temp. and pH was adjusted to 1.0 with 2M HCl and kept at 27-28°C for 3-days. This solution was lyophilized and loaded on preparative HPLC.

The mobile phase used was as follows:

Buffer	: Aqueous acetic acid 0.01%
Solution A	: Buffer + Acetonitrile in ratio of (85:15)
pH	: As such
Wavelength	: 254nm
Flow rate	: 20 ml/min
Column	: Inertsil ODS 3-V (250mm x 20mm and 08μm particle size).

The fractions containing the impurity were collected and lyophilized. The solid mass was taken for further evaluation of the purity and other spectroscopic studies.

The PMR spectrum was recorded in DMSO d_6. The mass spectrum was recorded by dissolving sample in acetonitrile/water using Atmospheric pressure ionization having turbolon ionization source. The declustrring potential was set at 10, focusing potential at 80, and ionization volt energy (IVE) 4500 at 400°C was used.

Conclusion

The structure of impurities B and B1 were assigned as distereoisomers formed due to lactonization between 2-carboxyl and 3-vinyl group, βlactam ring opening and the elimination of carboxylic group.

PMR Spectra

The PMR spectra in DMSO d6 confirm the structure of above-mentioned impurity

S.No.	Chemical shift (ppm)	Multiplicity	Assignment
1)	1.35	d	$-CH_3$
2)	3.44	m	$-7-CH_2$
3)	3.54	q	SCH_2
4)	4.54	d	6-H
5)	5.12	q	Lactone CH
6)	6.02	d	-NH
7)	6.79	s	H of Ar
8)	7.08	br. s	NH_2
9)	8.58	t	CONH
10)	11.24	br. s	NOH

The above spectral data shows vinyl proton signals of cefdinir disappeared and two signals assigned to the $-CHCH_3$ moiety were found, suggesting that it contain γ-lactone derived from lactonization between 2-carboxyl and 3-vinyl group.

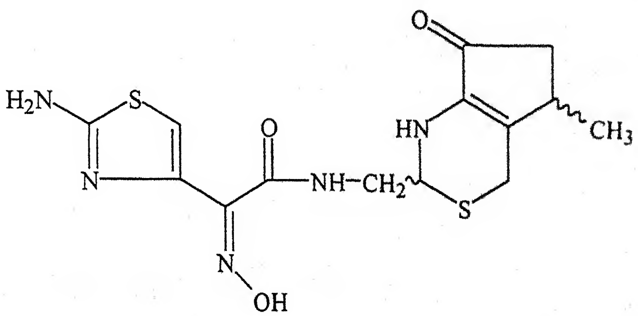
Mass Spectra

The assignment to the molecular ion peak is given below:

S.N.	m/z amu	Structural
1.	370 (M+H)+	Molecular ion

Summary

The unidentified impurities B and B1 were isolated from Cefdinir using MPLC and preparative HPLC. The structure of these impurities were assigned on the basis of PMR and Mass spectra. They were characterized as isomer of (Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino)-N- [[(2RS, 5RS)-1,2,5,7-tetrahydro-5-methyl-7-oxo-4H furo- [3,4-d][1,3] thiazin-2-yl] methyl] acetamide.



Isolation of cefdinir impurity - D

Charged cefdinir #08006 (05 gm) in round bottom flask Followed by water (100 ml) and heated at 90°C for 3.0 hrs. The impurity enrichment was about 20%; this solution was loaded on preparative HPLC. The mobile phase used was as follows:

Buffer	: 0.1M ammonium acetate + ACN in the ratio of 90:10
pH	: As such
Wavelength	: 254nm

Flow rate : 15 ml/min

Column : Inertsil ODS 3-V (250mm x 20mm and 08µm particle size).

The fractions containing the impurity were collected and lyophilized. The solid mass was taken for further evaluation of the purity and the spectroscopic studies.

The PMR spectrum was recorded in DMSO d_6. The mass spectrum was recorded by dissolving sample in acetonitrile/water using Atmospheric pressure ionization having turbolon ionization source. The declustrring potential was set at 10, focusing potential at 80, and ionization volt energy (IVE) 4500 at 400°C were set.

Conclusion

The structure of impurity was found as E-isomer of cefdinir .

PMR Spectra

The PMR spectra in DMSO d6 confirm the structure of above-mentioned impurity.

S.No.	hemical shift (ppm)	Multiplicity	Assignment
1)	3.57-3.84	q	SCH_2
2)	5.18	d	6-H
3)	5.32	d	Vinyl
4)	5.60	d	Vinyl
5)	6.11	q	7-H
6)	6.93	dd	Vinyl
7)	7.33	br. s	NH_2
8)	7.53	s	Ar
9)	9.38	d	CONH
10)	12.90	br. s	NOH

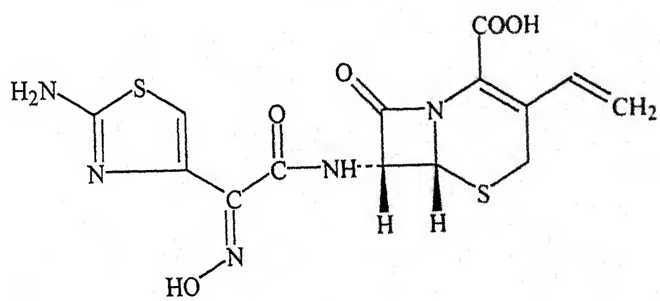
Mass Spectra

The assignment to the molecular ion peaks and their fragments are given below:

S.No.	m/z amu	Structural
1	396 (M+H)+	Molecular ion

Summary

The unidentified impurity D was isolated from Cefdinir using MPLC and preparative HPLC. The structure of this impurity was assigned on the basis of PMR and Mass spectra. It was characterized as (6R, 7R)-7-[(E)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino) acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid.



Isolation of cefdin impurity- E

Charged cefdinir #08006 (05 gm) in round bottom flask Followed by water (100 ml) and adjusted pH 12 with 8m NaOH and stirred at 26-27°C for 4.0 hrs. by keeping reaction flask covered with black paper

thereafter added 1.5 gm NaCl and pH was adjusted to 5.5 with 5 M HCl. This solution was loaded on MPLC.

The conditions used was as follows:

Solution A : 1.5% Aqueous NaCl

Solution B : 1.5% Aqueous NaCl + Acetonitrile in ratio of (85:15)

pH : As such

Wavelength : 254nm

Flow rate : 100-ml/ min

Column : 700mm x 50mm diameter packed with C18 silica of 20-40μm

MPLC was used for initial enrichment of the impurity sample up to about 25% (by HPLC area method). The following gradient program was used:

Time	Event	Value	Flow ml/min
00.01	B.conc	00.0	150.00
30.00	B.conc	00.0	150.00
45.00	B.conc	50.0	150.00
60.00	B.conc	100.0	150.00
70.00	B.conc	100.0	150.00
80.00	B.conc	00.0	150.00
95.00	B.conc	00.0	150.00
96.00	B.conc	00.0	Stop

All fractions collected were monitored using the above analytical method. The fractions containing impurity were concentrated using rotavapour at bath temperature 30-35°C in high vacuum to distill the Acetonitrile.

The solution was lyophilized, and thereafter loaded on preparative HPLC for further purification.

Purification of lyophilized fractions of MPLC by preparative HPLC

The mobile phase used was as follows:

Solution A : Water 100%

Solution B : Water + Acetonitrile in ratio of (60:40)

pH : As such

Wavelength : 254nm

Flow rate : 15 ml/min

Column : Inertsil ODS 3-V 250mm x 20mm, 08μm particle size

The following gradient program was used:

Time	Event	Value	Flow ml/min
00.01	B.conc	00.0	15.00
18.00	B.conc	00.0	15.00
24.00	B.conc	100.0	15.00
35.00	B.conc	100.0	15.00
40.00	B.conc	00.0	15.00
45.00	B.conc	00.0	15.0
50.00	B.conc	00.0	Stop

The fractions containing the impurity were collected and were concentrated using rotavapour at bath temperature 30-35°C in high vacuum to distill out the Acetonitrile and remaining aqueous part was lyophilized. The solid mass was taken for further evaluation of the purity and the spectroscopic studies.

The PMR spectrum was recorded in DMSO d_6 .The mass spectrum was recorded by dissolving sample in acetonitrile/water using

atmospheric pressure ionization having turbolon ionization source. The declustrring potential was set at 10, focusing potential at 80, and ionization volt energy (IVE) 4500 at 400°C were set.

Conclusion

The impurity assigned as β-lactam ring opened cefdinir, which was formed by base catalyzed hydrolysis.

PMR Spectra

The PMR spectra in DMSO d6 confirm the structure of Above mentioned impurity

S.N.	**Chemical shift (ppm)**	**Multiplicity**	**Assignment**
1	1.85	t	CH_3
1	3.57-3.84	q	SCH_2
1	5.37	d	7-H
1	6.28	q	Vinyl
1	7.28	s	Ar
1	7.53	s	NH2
1	9.38	D	CONH

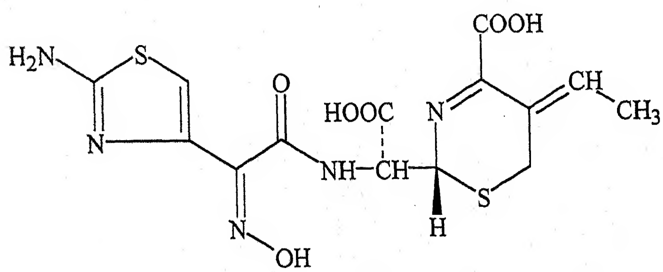
Mass Spectra

The assignment to the molecular ion peaks and their fragments are given below:

m/z amu	Structural
413.43 (M+H)+	Molecular ion

Summery

The unidentified impurity E was isolated from Cefdinir using MPLC and preparative HPLC. The structure of this impurity was assigned on the basis of PMR and Mass spectra. It was identified as (R)-2-[(Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino) acetamido]-(2R)-4-carboxy-5-[(Z)-ethylidine]-5,6-dihydro-2H-thiazin-2-acetic acid.



Area % Report

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User: System
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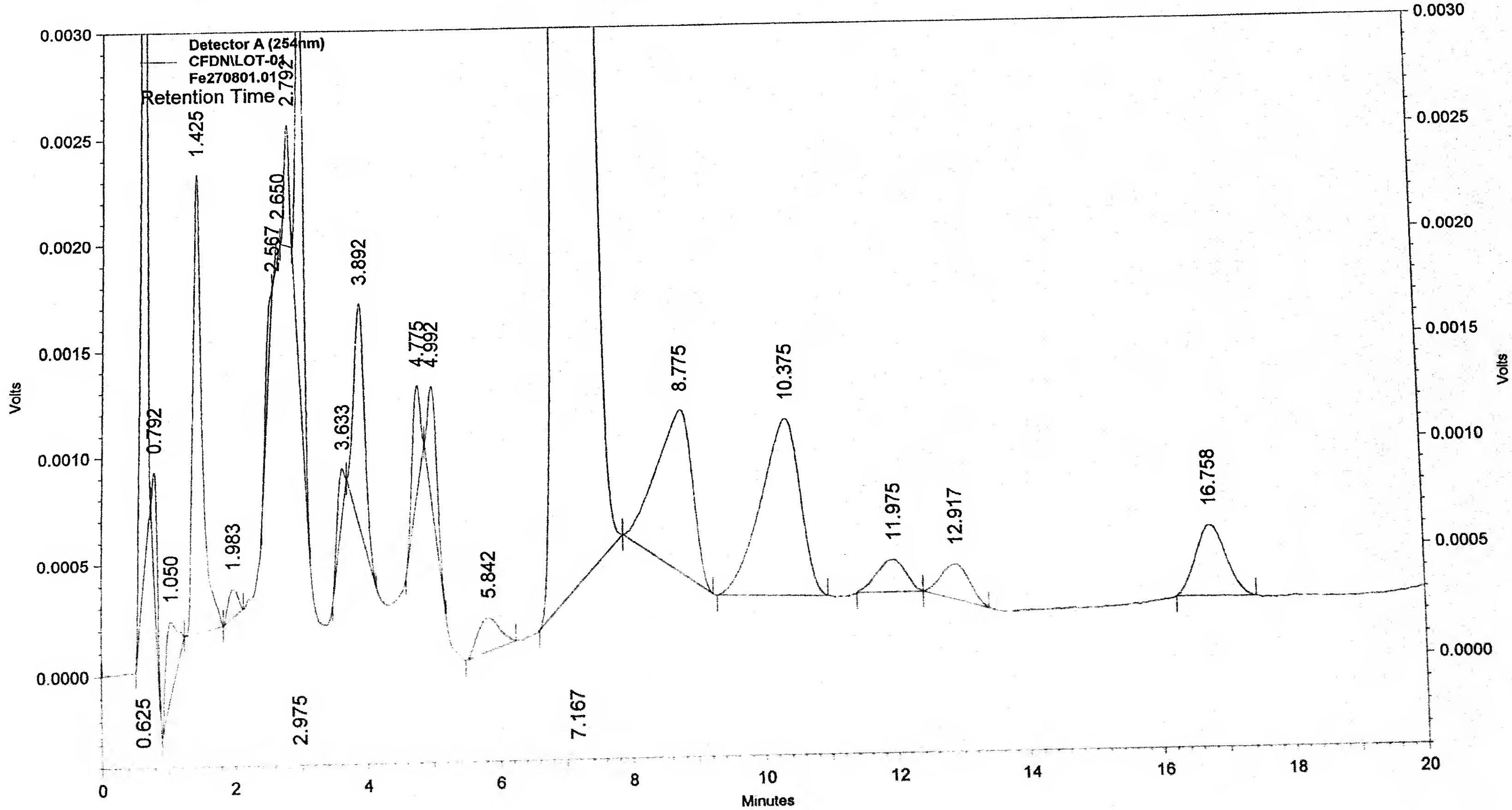

Area % Report

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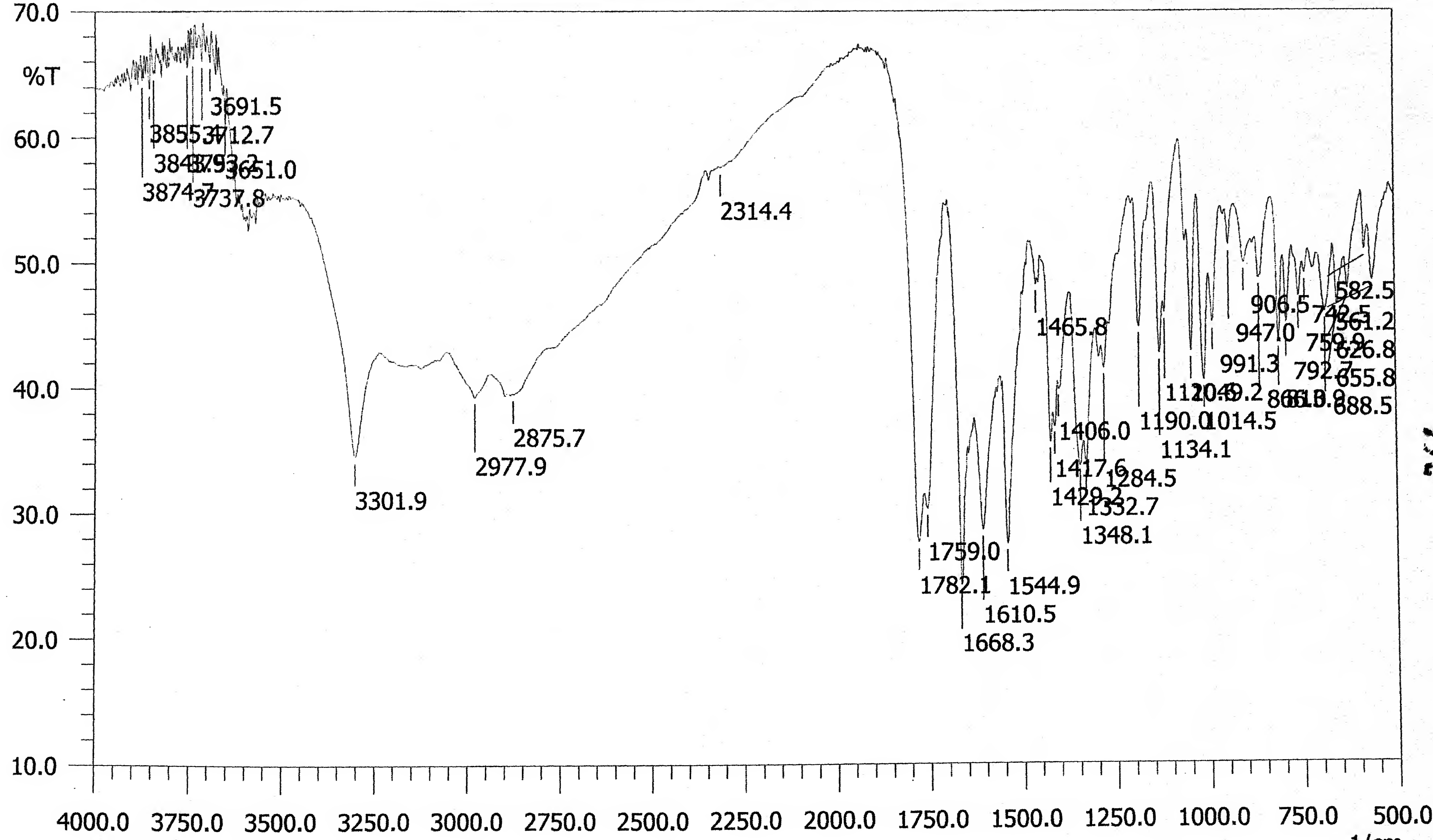
User: System
Acquired: 8/27/01 4:05:24 PM

Detector A (254nm)

Pk #	Retention Time	Area	Area %
1	0.625	26336	1.318
2	0.792	2946	0.147
3	1.050	3971	0.199
4	1.425	20280	1.015
5	1.983	1294	0.065
6	2.567 – A	1548	0.077
7	2.650 – A1	440	0.022
8	2.792 – A2	2926	0.146
9	2.975 – E	15069	0.754
10	3.633	1852	0.093
11	3.892	11701	0.585
12	4.775 – B	4335	0.217
13	4.992 – B1	5170	0.259
14	5.842	3464	0.173
15	7.167 – CEFDINIR	1815223	90.828
16	8.775 – C	28487	1.425
17	10.375 – C1	33638	1.683
18	11.975 – C2	4518	0.226
19	12.917 – C3	4845	0.242
20	16.758 – D	10419	0.521
21	30.408	69	0.003
Totals		1998531	100.000

243

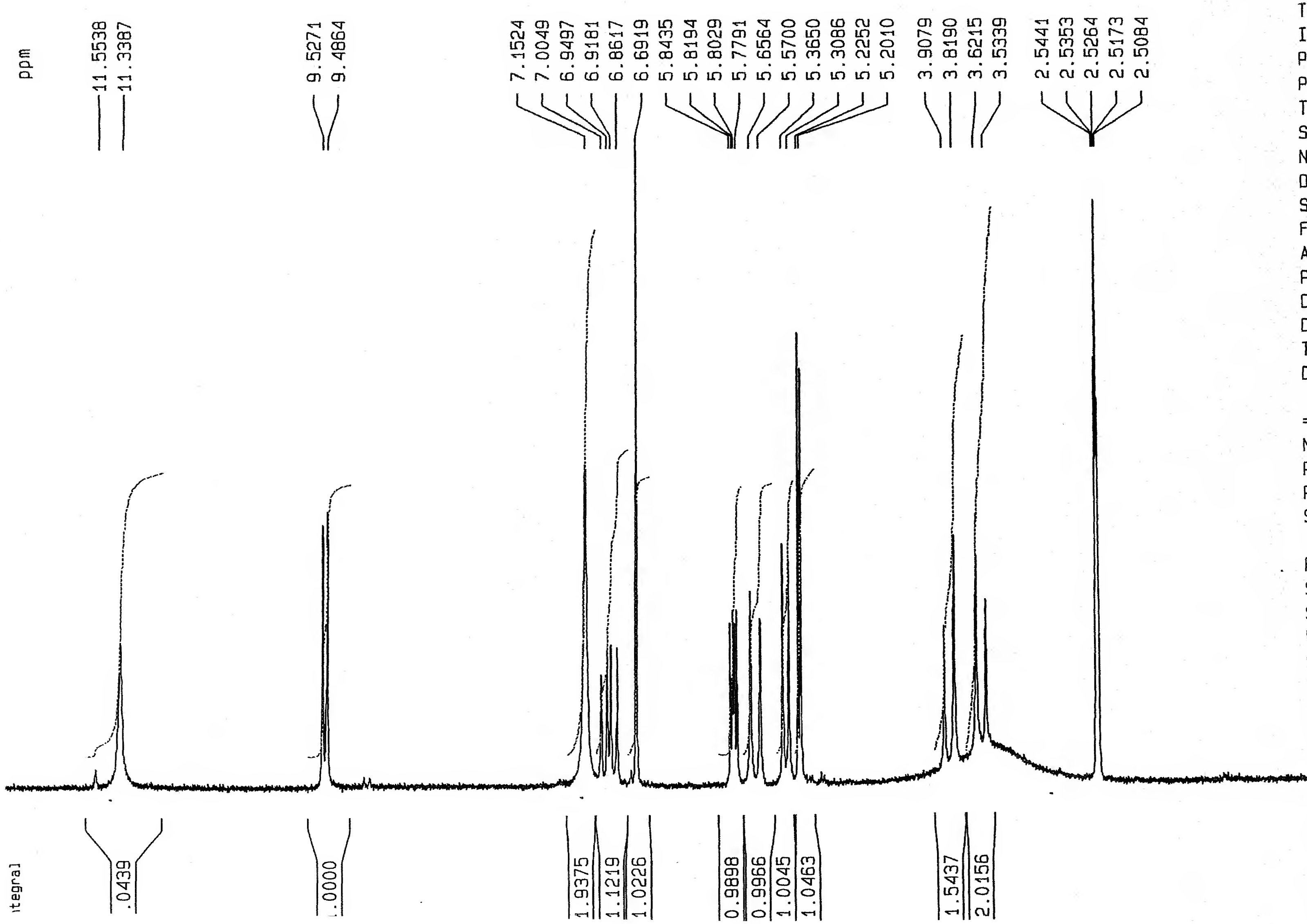


Cefdinir W.S.006

CFD.IRS: Cefdinir W.S.006

Date:	04/16/2002	Time:	08:55:45	NScans:	4
Type:	HYPER IR	User:	LUPIN RESEARCH PARK	Detector:	standard
Abscissa:	1/cm	Ordinate:	%T	Apodization:	Happ
Min:	401.17	Max:	3998.16	Range:	1/cm

Proton NMR Spectra of Cefdinir Working STD-006 in DMSO-d6



Current Data Parameters

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EXPNO	50
PROCNO	1

F2 - Acquisition Parameters

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PULPROG	zg30
TD	32768
SOLVENT	DMSO
NS	32
DS	0
SWH	2997.602 Hz
FIDRES	0.091480 Hz
AQ	5.4657526 sec
RG	574.7
DW	166.800 usec
DE	7.11 usec
TE	300.0 K
D1	2.00000000 sec

======== CHANNEL f1 ========

NUC1	1H
P1	6.00 usec
PL1	-5.00 dB
SFO1	200.1314009 MHz

F2 - Processing parameters

SI	16384
SF	200.1300000 MHz
WDW	SINE
SSB	2.5
LB	0.00 Hz
GB	0
PC	3.00

1D NMR plot parameters

CX	21.00 cm
CY	16.00 cm
F1P	12.349 ppm
F1	2471.48 Hz
F2P	0.628 ppm

SUMMARY

This investigation was aimed to develop stability indicating method for cefixime and cefdinir, validation of their assay method, isolation of impurities, which are exist 0.1% in amount both at initial stage, and after accelerated conditions and finally to check their resolution with cefixime and cefdinir main peaks. The findings are summarized below:

A. Cefixime assay method validation.

Accuracy: Accuracy calculated by recovery method, and average recovery is 99.74% which is well within specified range (i.e. Recovery should be within 100 ± 1.5 %)

Precision: Precision is calculated based on the Relative standard Deviation (RSD) of Retention time (RT) and Area of six replicate injections. The observed RSD of RT is 0.15%, 0.16%, and 0.023% and Area is 0.068%, 0.07%, and 0.161%, at 50%, 100%, and 150% concentration which is also within specified range (i.e.RSD of RT and area should not be more than 1.0%).

Linearity: Linearity is checked in the range of 150% and 50% of target concentration, and coefficient of correlation is found 0.9999%. Which is also within specified limit.(i.e. coefficient of correlation should not be less than 0.999%).

Range: Range is determined from RSD of RT and area of Cefixime peak at 150% and 50% concentration. RSD of RT and area of cefixime at 150% is 0.023% and 0.161% respectively and at 50% concentration RSD of RT and area is 0.15% and 0.068%. This is

Also within specified limit (i.e. RSD of RT and area should not be more than 1.0%).

Specificity: It is calculated based on the theoretical plate, Tailing factor and Resolution of cefixime and E-isomer of cefixime. Observed resolution is 3.16 between cefixime and E-isomer of cefixime, tailing factor is 1.09 and 1.69 for E-isomer and cefixime respectively and theoretical plate is 9391.78 and 6741.65 for E-isomer and cefixime respectively. This is also within specified limit. (i.e. Theoretical plate not less than 5000 for E-isomer and 4500 for cefixime, resolution not less than 2.0, and tailing factor not more than 2.0).

Robustness: It defined as " a measure of analytical method's capacity to remain unaffected by small, but deliberate variations in method parameters" like change in flow rate, change in pH, change in composition of buffer, and change in organic composition etc. Cefixime method found robust against all the above-mentioned parameters, as resolution, tailing factor and theoretical plate are within acceptable range. (I.e. Resolution between cefixime and E-isomer is not less than 2.0, theoretical plate of cefixime and E-isomer are not less than 4500, and 5000 receptively, and tailing factor is not more than 2.0).

Stability of sample: To generate reproducible and reliable results, the samples, standards, and reagents used for the HPLC method must be stable for a reasonable time (e.g. one day, one week, one month, depending on need). For cefixime, sample in mobile phase found stable up to 12 hrs. At 25°c.

Forced degradation: During stressed condition, non-of the impurity found to be merged with main cefixime peak. Checked by spectrum correlation and contour plot of Photo diode Array detector.

B. Cefdinir assay method validation.

Accuracy: Accuracy calculated by recovery method, and average recovery is 99.93% which is well within specified range (i.e.% Recovery should be within 100 ± 1.5 %).

Precision: Precision is calculated based on the Relative standard Deviation (RSD) of Retention time (RT) and Area of six replicate injections. The observed RSD of Area is 0.29%, 0.14%, and 0.07% and RT is 0.001%, 0.05%, and 0.03%, at 50%, 100%, and 150% concentration which is also within specified range (i.e. RSD of RT not more than1.5% and area not be more than 1.0%).

Linearity: Linearity is checked in the range of 150% and 50% of target concentration, and coefficient of correlation is found 0.9999%. Which is also within specified limit.(i.e. coefficient of correlation should not be less than 0.999%).

Range: Range is determined from RSD of RT and area of Cefdinir peak at 150% and 50% concentration. RSD of RT and area of cefdinir at 150% is 0.03% and 0.07% respectively and at 50% concentration RSD of RT and area is 0.001% and 0.29 %. This is also within specified limit (i.e. RSD of RT not more than1.5% and area not be more than 1.0%).

Specificity: It is calculated based on the theoretical plate, Tailing factor and Resolution of cefdinir and impurity of cefdinir. Observed resolution is 9.10 between cefdinir and impurity of cefdinir, tailing

Factor is 1.07 for cefdinir and theoretical plate is 2503. This is also within specified limit. (I.e. Theoretical plate not less than 2000, resolution not less than 4.0, and tailing factor not more than 2.0).

Robustness: It defined as " a measure of analytical method's capacity to remain unaffected by small, but deliberate variations in method parameters" like change in flow rate, change in pH, change in composition of buffer, and change in organic composition etc. Cefdinir method found robust against all the above mentioned parameters, as resolution and theoretical plate are within acceptable range. (i.e. Resolution between cefdinir and impurity is not less than 4.0,tailing factor not more than 2.0 and theoretical plate of cefdinir is not less than 2000).

Stability of sample: To generate reproducible and reliable results, the samples, standards, and reagents used for the HPLC method must be stable for a reasonable time (e.g. one day, one week, one month, depending on need). For cefdinir, sample in mobile phase found stable up to 12 hrs. at 25°c.

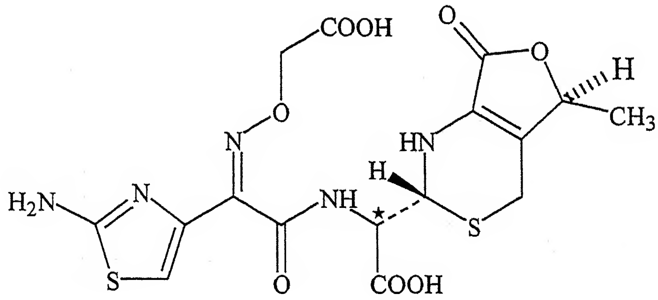
Forced degradation: During stressed condition, non-of the impurity found to be merged with main cefdinir peak. Checked by spectrum correlation and contour plot of Photo diode Array detector.

CEFIXIME IMPURITIES

Following impurities were isolated from Cefixime using Medium Pressure Liquid Chromatography and Preparative HPLC and characterized.

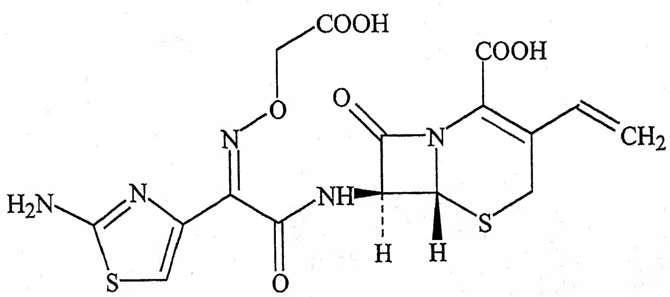
1. Impurity – A of cefixime

2-((2R)-5-methyl-7-oxo(5-hydro-1H,2H,4H-furano[3,4-d]1,3-thiazin-2-yl))(2S)-2-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy)prop-2-enoylamino acetic acid



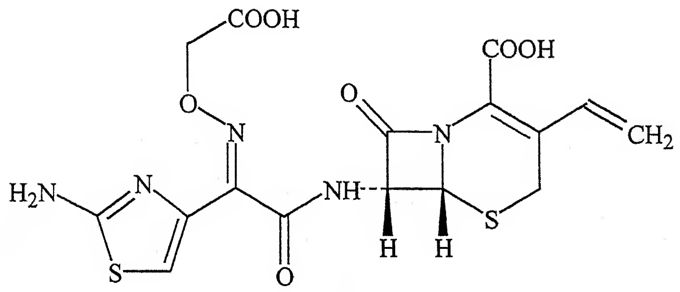
2. Impurity – C of cefixime

(7S)-7-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy) prop-2-enoylamino]-1-aza-8-oxo-5-thia-3-vinylbicyclo[4.2.0]octane-2-carboxylic acid



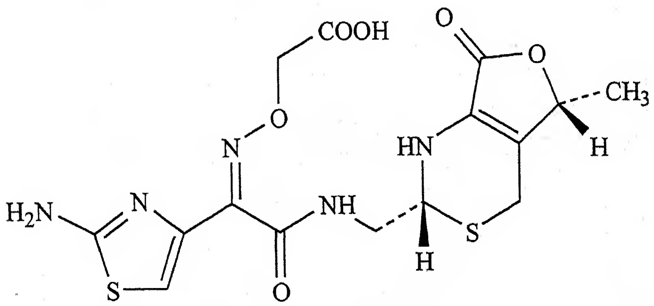
3.Impurity – D of cefixime

7-[(2E)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy)prop-2-enoylamino](7R)-1-aza-8-oxo-5-thia-3-vinylbicyclo[4.2.0]octane-2-carboxylic acid



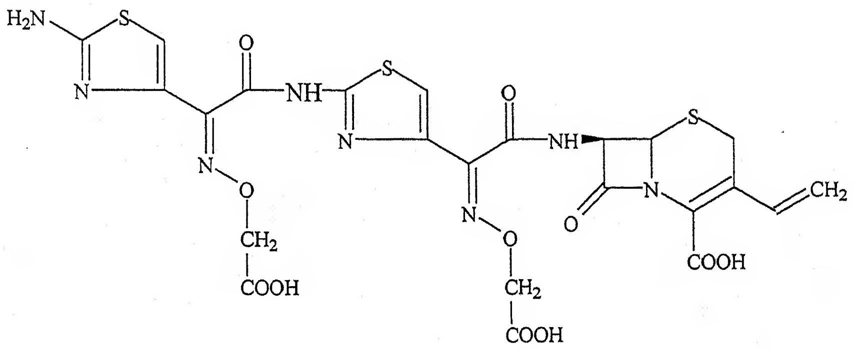
4.Impurity – B of cefixime

2-(2-{N-[((2R,5R)-5-methyl-7-oxo(5-hydro-1H,2H,4H-furano[3,4-d] 1,3-thiazin-2-yl))methyl]carbamoyl}(1Z)-2-(2-amino(1,3-thiazol-4-yl) -1-azavinyloxy)acetic acid



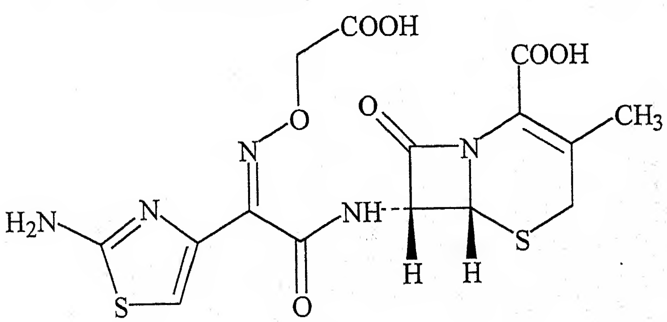
5. Impurity – F of cefixime

(6R)-6-((2Z)-2-{2-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy) prop-2-enoylamino](1,3-thiazol-4-yl)}-3-aza-3-(carboxymethoxy)prop-2-enoylamino -5-oxo-3-vinyl-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid



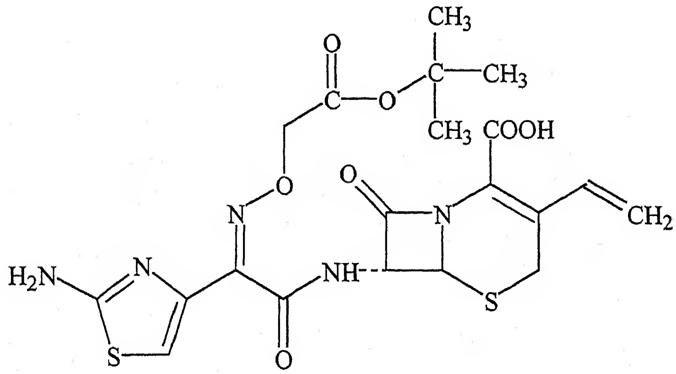
6.Impurity – E of cefixime

(6R)-6-[(2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-(carboxymethoxy)prop-2-enoylamino]-3-methyl-5-oxo-2H,6H,6aH-azetidino[2,1-b]1,3-thiazine-4-carboxylic acid



7.Impurity – I of cefixime

(6R)-6-((2Z)-2-(2-amino(1,3-thiazol-4-yl))-3-aza-3-{[(tert-butyl)oxycarbonyl methoxy}prop-2-enoylamino)-5-oxo-3-vinyl-2H,6H,6aH-azetidino[2,1-b]1,3 -thiazine-4-carboxylic acid

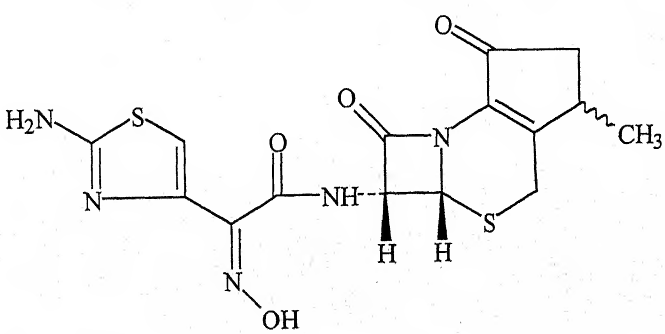


CEFDINIR IMPURITIES

Following Cefdinir impurities were isolated from Medium Pressure Liquid Chromatography and Preparative HPLC and characterized.

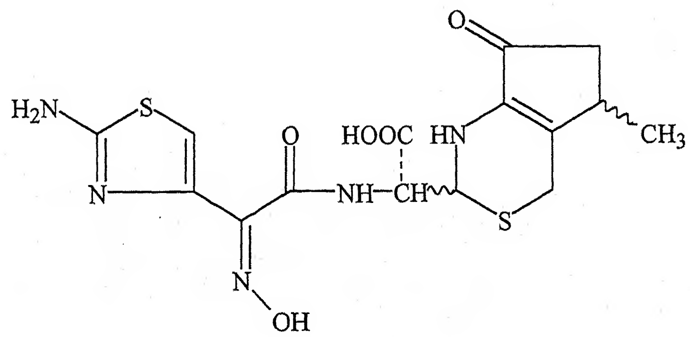
1.Impurity – A of cefdinir

(Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino)-N-[(3RS,5aR,6R)-1,4,5a,6-tetrahydro-3-methyl-1,7-dioxo-3H,7H-azeto[2,1-b]furo-[3,4-d][1,3]thiazin-6-yl]acetamide



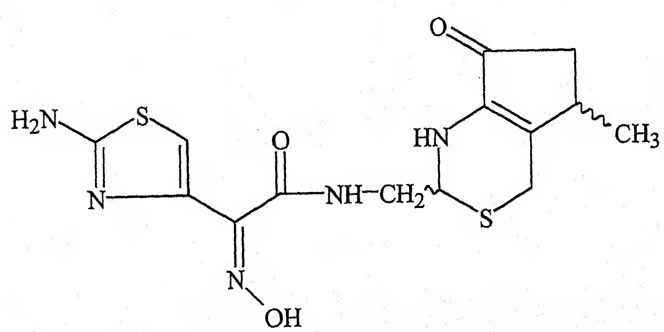
2.Impurity – C of cefdinir

(R)-2-[(Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino)acetamido]-2-[(2RS,5RS)-1,2,5,7-tetrahydro-5-methyl-7-oxo-4H-furo[3,4-d]-[1,3] thiazin-2-yl]acetic Acid.



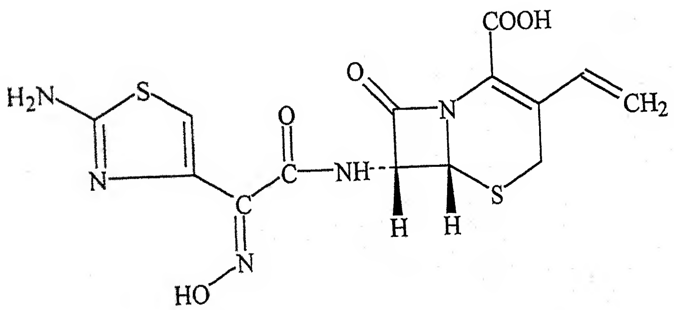
3.Impurity – B of cefdinir

(Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino)-N- [[(2RS, 5RS)-1,2,5,7-tetrahydro-5-methyl-7-oxo-4H furo- [3,4-d][1,3] thiazin-2-yl] methyl] acetamide.



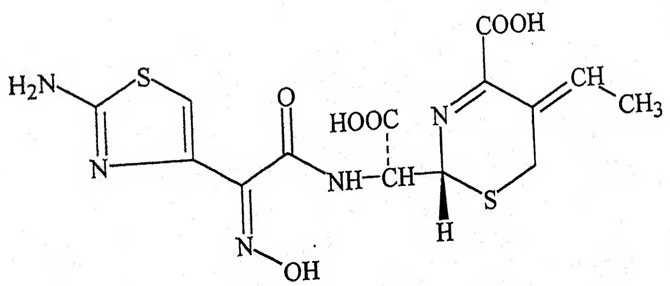
4.Impurity – D of cefdinir

(6R, 7R)-7-[(E)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino) acetamido]-8-oxo-3-vinyl-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid.



5.Impurity – E of cefdinir

(R)-2-[(Z)-2-(2-Amino-4-thiazolyl)-2-(hydroxyimino) acetamido]-(2R)-4-carboxy-5-[(Z)-ethylidine]-5,6-dihydro-2H-thiazin-2-acetic acid.



This study would help in submitting the data in Drug Master file (DMF) and Abbreviated new drug application (ANDA) required for developed markets such as USA, Europe, Canada etc.